IMMUNITY TO HISTOPLASMOSIS INDUCED IN MICE BY COMPONENTS OF *HISTOPLASMA CAPSULATUM*

by

Kenneth Leroy Anderson

A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Microbiology

University of Utah

August, 1968

This Thesis for the

Doctor of Philosophy Degree

by

Kenneth Leroy Anderson

has been approved

[redacted] Supervisory Committee

[redacted]

[redacted]

[redacted]

[redacted] Supervisory [redacted]

[redacted]

[redacted]

[redacted]
Head, Major Department

[redacted]
Dean, [redacted]

I am deeply indebted to Dr. Stanley Marcus who has critically reviewed this manuscript and for his assistance, guidance, and encouragement during these studies.

Grateful acknowledgement is made to Dr. Louis P. Gebhardt, Dr. Paul S. Nicholes, Dr. Bill B. Wiley, Dr. Douglas W. Hill, Dr. Gilbert A. Hill, and Dr. Charles C. Nabors who have made extensive and important suggestions during my association at the University of Utah.

Special thanks is extended to Dr. Thomas F. Dougherty and Dr. Marvin Rogolsky for their help and interest.

Particular recognition is extended to numerous colleagues for their encouraging words during these studies and to my wife, Jeanne, without whom this would have been impossible.

The author wishes to thank Mrs. Diane Ward, Mrs. Helen Wyatt, and Miss Arlene Imada for help in typing and proof-reading of this thesis.

Special appreciation goes to Mrs. Priscilla Maydens, Medical Librarian, who aided the author in the format and in checking the bibliographical material.

The research reported herein has been supported by Public Health Service Grant (AI-K6-14,924) from the National Institutes of Health.

TABLE OF CONTENTS

TABLES

FIGURES

ABSTRACT

Twenty-three strains of Histoplasma capsulatum were studied. The data indicated that each strain had individual characteristics with regard to: macroscopic appearance of colonies, measurement of spore sizes, relative number of spores, virulence as determined by intravenous LD_{50} in mice, immunization capacity of vaccines, and chemical composition of the vaccines prepared from four strains. Strains G10, G17M, 2645, and 3330 were used to study the immunogenicity of the fungus. A cell free extract prepared from the mycelial phase of strain G10 and a cell free extract prepared from the yeast phase of strain G17M produced similar protection in immunized mice. G10 and G17M vaccines also had similar chemical composition with high carbohydrate content and low protein content. Strains 3154 and G17M were found to be the most virulent by the method employed. The sporulation number, as determined by a rank rating system, showed that each strain produced various numbers of both types of spores. The results indicated that Sabouraud's medium with phosphate ion produced abundant and numerous spores. The macroscopic description of each strain on various media indicated that each strain varies in morphological characteristics depending on the nutrition available.

IMMUNITY TO HISTOPLASMOSIS INDUCED IN MICE BY COMPONENTS OF *HISTOPLASMA CAPSULATUM*

INTRODUCTION

The extent and mechanisms of resistance to histoplasmosis represent an area of significant basic and applied effort in mycology. The problems associated with the antigenic analysis of *Histoplasma capsulatum* are as complex as the fungi themselves. In its natural habitat of soils or on agar incubated at room temperature, the fungus forms an intricate network of interlaced hyphae, from which a specialized complex of spores are produced. The large, globose macroconidium, characteristic of *H. capsulatum*, has a thick wall, internal structure, and many short, finger-like appendages emanating from all sides. In addition there exists a second type of spore called the microconidium. The smooth-walled microconidium is spherical or pyriform in shape. It is possible that the constitution of the macroconidium is not identical with that of the microconidium. Two other elements exist which may also be different in composition: the yeast phase of the organism and the hyphal elements.

The objectives of the work presented in this thesis have been: a) preparation of vaccines from various elements

of *H. capsulatum* in order to determine immunogenic capacity; b) characterization of the virulence of selected yeast phase strains of the organism by intravenous LD_{50}; c) differential spore production on various media; and d) description of the macroscopic appearance of strains of the fungus on various media.

REVIEW OF LITERATURE

Increased awareness in recent decades of the prevalence of airborne fungi and the high frequency of these organisms as etiological agents of pulmonary disease has stimulated wide-spread interest in their pathogenicity and immunogenicity. The defense mechanisms are little understood in the mycoses. Antibodies arising during the course of disease have high diagnostic and prognostic values but the protective effect of such antibodies has not been demonstrated. Cellular responses to infection with these organisms produce granulomas and delayed hypersensitivity. Such reactions are salient features, the former is associated with heightened immunity but the role of the latter is undetermined.

I. HISTORY OF HISTOPLASMOSIS

The causal agent of histoplasmosis was first observed by Darling (1906, 1908, 1909). He believed the organism to be a protozoan and gave it the generic name, Histoplasma. The species name, capsulatum, was used to describe a capsular-appearing halo surrounding the organism in stained tissue sections. Da Rocha-Lima (1912) was the first to suspect that the organism was actually a fungus and not a protozoan. H. capsulatum was obtained in culture by Hansmann and Schenken in 1934 and a complete description of the

organism was published by De Monbreun in 1934. De Monbreun clearly demonstrated the diphasic nature of the fungus and accurately described the cultural characteristics, morphology and life cycle of the organism. Ciferri and Radaelli (1934) stated the systematic position of H. capsulatum as follows: genus Histoplasma, order Blastosporales, superfamily Atelosaccharomycetaceae, and family Histoplasmaceae. Since no sexual stage could be demonstrated for H. capsulatum, Conant (1941) placed the organism in the Fungi Imperfecti, as a member of the form-family Moniliaceae. However sexual reproduction has been demonstrated by Ajello and Cheng (1967). The development of an ascigenous state was stimulated on soil overlaid with feathers. The organism is homothallic and is given the perfect state name of Gymnoascus demonbreunnii.

A diagrammatic representation of the life cycle of H. capsulatum and G. demonbreunnii is shown in Figure 1. The three cycles represent the mycelial and yeast phase of the asexual H. capsulatum and the sexual stage of the fungus G. demonbreunnii. The various structures, forms, and spores are depicted. Recently the validity of the species G. demonbreunnii as the perfect state of H. capsulatum has been questioned (Kwon-Chung, 1968).

Knowledge of the growth of H. capsulatum in nature dates from the first isolation by Emmons, Morlan and Hill

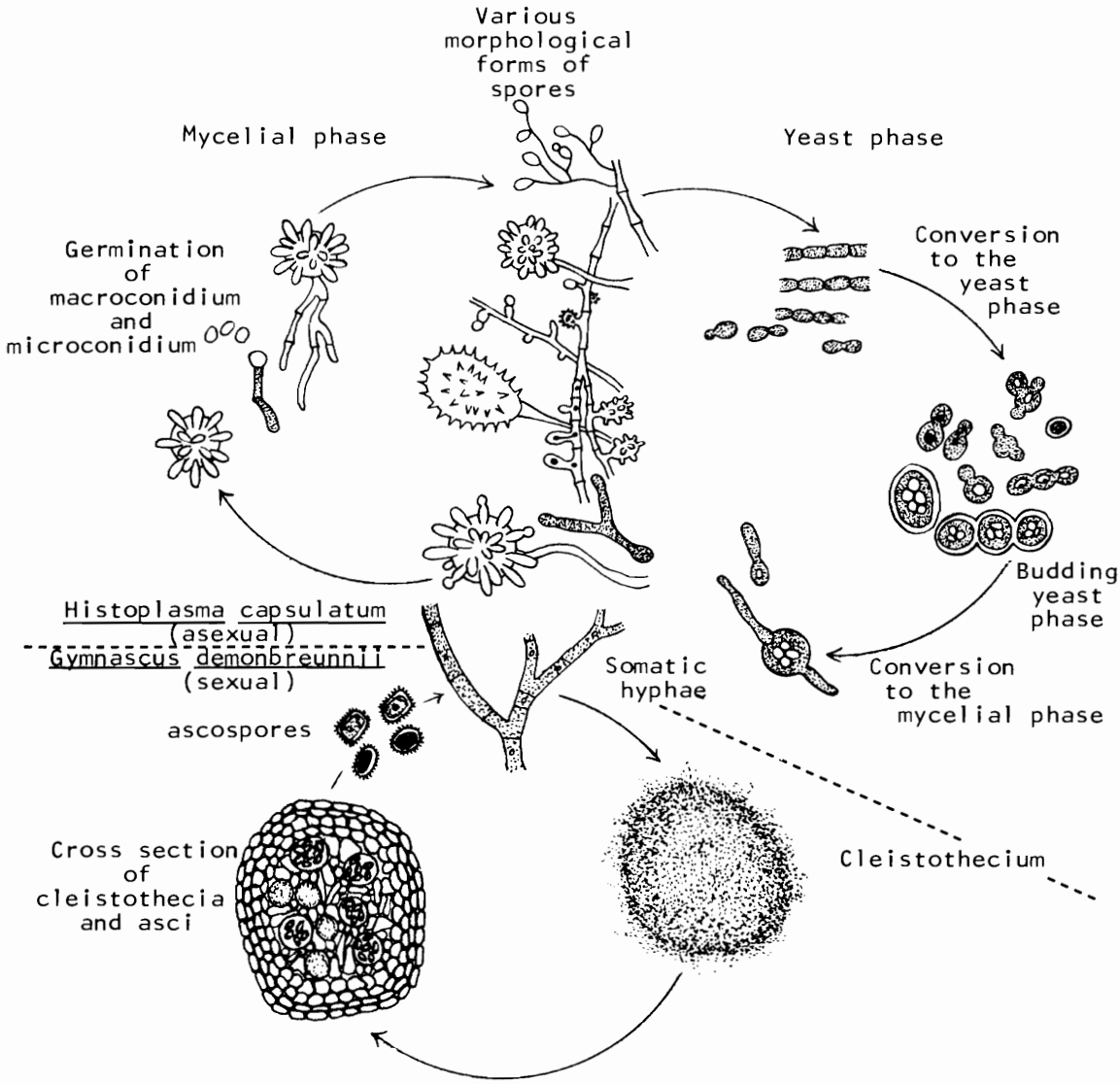

Figure 1. A diagrammatic representation of the life cycle of Histoplasma capsulatum and Gymnascus demonbreunnii

(1949). They isolated the organism from the soil about a rodent burrow under a chicken coop. Grayston, Loosli, and Alexander (1951) reported the first isolation of the organism in connection with an epidemic of histoplasmosis which occurred in a silo in Northern Indiana. This fungus grows in the soil and is inhaled after the soil is stirred by some activity which creates an aerosol. In general, these organisms are small, 5 u or less in size (Cozad and Furcolow, 1953). Thus, in an aerosol these organisms can readily penetrate to the alveolar bed and be retained there (Brown, Cook, Ney, and Hatch, 1950). Dodd and Tompkins in 1934 described the first antemortem description of histoplasmosis. Following the publication of this work, fatal cases were recognized with increasing frequency. However, it was still regarded as a rare and fatal human disease.

In the 1940's a series of events conspired to change this situation. Christie and Peterson (1945) reported on pulmonary calcification with negative tuberculin tests in the Mississippi Valley area. Palmer (1945) incorporating histoplasmin skin testing in a broad survey on tuberculin sensitivity demonstrated a strong epidemiologic correlation between the incidence of pulmonary calcifications and positive histoplasmin skin test in individuals with negative tuberculin skin tests. These discoveries permitted a new

evaluation and understanding of the epidemiology of the disease produced by H. capsulatum.

Histoplasmosis has been found to be an intracellular mycosis of the reticuloendothelial system. Usually respiratory in origin, histoplasmosis may be asymptomatic or benign, acute or chronic, and widely disseminated or fatal. H. capsulatum parasitism of reticuloendothelial cells probably results in early and wide dissemination even in mild or inapparent disease (Emmons, Binford and Utz, 1963).

Histoplasmosis is a disease of worldwide distribution. The hypothesis has been suggested that all of the river valleys in the temperate and tropical zones of the world, between 45° north and 45° south latitude are endemic areas (Furcolow, 1960). It has been estimated that in the United States alone 30 million people have been infected with this fungus, and half a million a year acquire the infection (Furcolow, 1965).

II. MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS

A. Mycelial Phase

When mycelial fragments are streaked over Sabouraud's dextrose agar, small white, cottony colonies develop which increase in size. At the time maximum growth is reached, the central portion and upper drier parts of the culture assume a yellow to buff color which slowly turns to tan or

dark brown. Gradually the entire culture will become brown in color and will take on a drier more fragile or powdery appearance. Usually sporulation is associated with the tan coloration and aging of the culture (Pine, 1960).

In general, the mycelium varies from 1 - 5 μ in diameter. The hyphae are usually refractile, branched, and multicellular (septate). Each mycelial cell component has one or more nuclei (Negroni, 1940). When the mycelium becomes older, its protoplasm is displaced toward the walls and vacuoles and oil globules are seen in the central portions of the cells. At this time, the walls thicken and a few cells may develop into peculiar appearing swellings of assorted shapes and sizes which in some cases reach a diameter as great as 15 μ. Racquet hyphae and occasionally coiled hyphae and nodular bodies may be formed while intercalary chlamydospores, single and in chains, and ranging from 5 - 8 μ in diameter may be found in most cultures. As the hyphae spread in radiating fashion from the center of the colony, parallel hyphae lying at the periphery may be observed to undergo anastomoses. These hyphal fusions do not result in the apparent formation of any specialized structures.

H. capsulatum produces two types of aleuriospores, macroconidia and microconidia (Howell, 1939). The macroconidia are typically large tuberculate, spherical to

pyriform spores described as ranging from 10 - 25 u (Pine, 1960), or 8 - 14 u (Emmons, Binford, Utz, 1963). These macroconidia are formed in the aerial portions of the mycelium generally at the end of short pedicels but may be sessile or formed at the end of long hyphae (Pine, 1960).

As described by Howell (1939) the large aerial spores begin their development as bulbous enlargements on the ends of lateral branches and are called spore initials. These branches may be simple with a single spore on the end of each branch, or one to several spores may be produced acropetalously and directly on a short branch. As these spore initials or prespores increase in size, the spores become spherical to pyriform in shape and their walls gradually increase in thickness.

The character of the spore wall varies greatly with the strain examined, the medium used, or the part of the mycelium in which it is produced (Howell, 1939). In addition to tuberculate and smooth macroconidia, there are forms having only several large bulbous or convoluted swellings or numerous spindly or warty projections (Negroni, 1940). Preponderantly smooth-walled macroconidia have been noted deep in the mycelial mat next to or embedded in the agar (Howell, 1939). Certain spores formed in these regions exhibit a halo of substance surrounding the body of the macroconidium and have

been called nymbospores (Nielsen and Evans, 1954). The spines or finger-like projections may be 1 - 8 u in length (Conant, 1941) but average about 5 u. It is the tuberculate spore which serves to identify the fungus.

The optimum pH for growth of the mycelial phase on a synthetic medium is approximately pH 6.5 (Howell, 1941). At pH 5.5 to 6.5 considerable aerial mycelium and macroconidia were formed on this medium whereas at pH 7.7 - 8.6 there was little or no aerial mycelium and sporulation was negligible. The optimum temperature for growth ranges from 25° to 30° C, and temperatures greater than 32° C are usually inhibitory (Howell, 1940). Based on optical density measurements, a generation time of approximately 12 hours has been obtained at 25° C in shake culture (Pine, 1954). A high humidity is recognized as usually being beneficial on agar media (Menges, Furcolow, Larsh and Hinton, 1952). The organism is a strict aerobe and growth will be greatly inhibited by merely closing off the culture tube with a rubber stopper or by attempting to grow the organism beneath the agar surface (De Monbreun, 1934).

Vitamin requirement studies of H. capsulatum show variable results. Scheff (1945) has reported H. capsulatum required biotin and niacin. However, Salvin (1949) studied different strains of the fungus and found that no vitamin supplement

was necessary. The amino acid requirement of the mycelial phase has been reported to be complex and variable for different strains (Rowley and Pine, 1955). Scheff (1945) obtained dry weights of mycelial mass with various carbohydrates; glucose, maltose and sucrose yielded the greatest weight increases.

Although the consensus of opinion is that the mycelial phase is readily grown at 25° C on a variety of media with relatively few nutritional requirements, the need for additional growth factors at 37° C is indicated (Howell, 1948; Scherr, 1956; 1957). The simplest basal medium for the growth of the mycelial phase is the glucose-asparagine synthetic medium used by Salvin (1949). However, generalizations should be guarded because each strain has its own peculiar characteristics regarding nutritional requirements, responses to temperature, and other environmental conditions.

B. Sporulation

Sporulation is dependent upon the strain of the organism and upon the environmental conditions of the culture. The addition of whole blood and temperatures greater than 32° C inhibit sporulation (Howell, 1940). Some strains sporulate better at pH values ranging from 6.5 to 7.5. Such increased sporulation may be correlated with greater growth. Negroni (1940) has shown that

sporulation may be greatly affected by the carbohydrate or nitrogen source in the medium. For example, in a glucose containing medium, asparagine supported best growth at room temperature, but only macroconidia were formed. When KNO_3 was substituted for asparagine as the nitrogen source less growth occurred and many smooth and rough macroconidia were formed. Utilizing $(NH_4)_2SO_2$ as the nitrogen source, this inorganic chemical resulted in little growth with only smooth macroconidia and microconidia being developed, and when no nitrogen source was added, Negroni found abundant microconidia and a few smooth macroconidia with scant mycelial growth.

Artis and Baum (1963) studied 32 strains of H. capsulatum focusing on the ability of these strains to form tuberculate spores. Nine strains did not produce tuberculate spores on Sabouraud's agar, on corn meal agar, on "spent" medium, or media with pH values ranging from 4.5 to 7.0. Tuberculate spore production did occur in these nine strains when Sabouraud's medium was enriched with phosphate, especially KH_2PO_4.

Smith and Furcolow (1964) suggested that the addition of infusions of starling manure to soil had the effect not only of producing more total particles but of producing a large number of microconidia and of increasing the viability

of these spores to at least twice that observed in other media. Of particular interest was the observation that although large numbers of particles are produced on Sabouraud's medium, 84% of these particles were hyphal elements and not spores, and the overall viability was only 3%.

Smith (1964) has reported that 0.6% yeast extract and 2% agar in distilled water stimulated rapid growth and sporulation of H. capsulatum similar to sporulation in starling manure extract medium. This rapidly grown fungus developed many viable microconidia despite a scant growth of vegatative mycelium.

H. capsulatum spores are resistant to drying and will remain viable in dry soil during an observation period of 4 years (Pine and Peacock, 1958). They are resistant to a temperature of 45° C for 30 minutes (Negroni, 1940). All spores of these strains were killed at a temperature of 50° C for 1 hour or at a temperature of 60° C for 5 minutes. The spores will survive over an observation period of 600 days in water at 4° C but rapidly decrease in viability as the temperature is increased to 37° C (Cooke and Kobler, 1953; Ritter, 1954).

C. The Yeast Phase

The yeast phase cells of young cultures are oval bodies which are approximately 1.5 to 2.0 u by 3.0 to 3.5 u

(Negroni, 1965). Budding occurs at either pole or may be apolar, with as many as three buds formed on a single mother cell. The cells usually appear round or oval, but elongated, swollen, or dumbbell shapes are also observed (Pine, 1960). The cells have a thin cell wall, the cytoplasm contains oil droplets and the nuclei of yeast cells appear peripherally as crescent shaped masses (De Monbreun, 1934). A halo about the cells appears to be a capsule, however India ink preparations do not reveal a true capsule (Conant, 1941). Ribi and Salvin (1956) were unable to find any electron-microscopic evidence for the presence of a capsule.

The optimum pH for yeast phase growth is considered to be between 6.5 and 7.5. Cross (1948) tested the effect of pH on the yeast-phase growth in three different media. Best growth was supported by brain-heart infusion broth at a pH range of 7.2 to 7.6. Salvin (1947) found that the maximum growth of the yeast-phase occurred between pH 6.3 and 8.1. On basal agar medium supplemented with whole blood, the rate of growth was similar at pH 5.5, 6.5, and 7.5 (Pine, 1954).

In general, the growth of the yeast phase on blood agar or serum media occurs at temperatures between 34° and 37° C. At lower temperatures conversion of the yeast phase to the mycelial phase occurs. However, growth of the yeast

phase of H. capsulatum of strains studied may be maintained at temperatures of 25° C (Pine, 1957; Scherr, 1956). Growth of the yeast phase is strictly aerobic (Pine, 1954). In liquid shake tubes the generation time ranges from 9 to 11 hours under optimal conditions (Pine and Peacock, 1958) while on blood agar media the generation time varies from 6 to 8 hours (Pine, 1955). Rowley and Huber (1956) inoculated mice with non-lethal doses of the yeast phase and estimated the generation time to be 15 to 19 hours. Howard (1964) determined the intracellular generation time in mouse histocytes to be 10.3 ± 1.5 hours.

As a carbon source, Negroni (1940) found glucose, mannose, or mannitol supported best growth. Salvin (1949) in a survey on the ability of the yeast phase to assimilate various nitrogen compounds found that only cysteine, cystine, and glutathione were assimilated. The vitamins necessary for growth were reported to be biotin (Salvin, 1949), thioctic acid (Pine, 1957), thiamine, inositol and niacin (McVeigh and Morton, 1965).

III. DEMONSTRATION OF IMMUNITY TO HISTOPLASMOSIS

A prerequisite for the demonstration of enhanced resistance is the use of a susceptible host. The subject of host range of H. capsulatum has been reviewed recently

(Salvin, 1963). The most widely used laboratory animal has been the mouse. The extent of immunity as measured by survival after challenge has been frequently employed, especially when high challenge doses are used. The mortality endpoint generally requires observation periods of several weeks to months. Marcus and Rambo (1952) reported on the determination of LD_{50} values for yeast phase cells injected intravenously and noted differences in virulence of two strains of H. capsulatum.

In growing bacteria and viruses, the organisms obtained are generally quite uniform in size and morphology. In contrast, cultures of Coccidioides immitis and H. capsulatum vary not only in size and shape but are often multiform in a given medium. With C. immitis for example, four distinct morphological forms may be found: 1) spherules, 2) endospores, 3) arthrospores, and 4) mycelial elements. Likewise H. capsulatum exists in four forms: 1) yeast phase cells, 2) mycelial elements, 3) macroconidia, and 4) microconidia.

It is now known that antigenicity varies with the morphological phase of different fungi. Kaufman and Blumer (1966) in studies with a fluorescent antibody for the yeast phase of H. capsulatum determined the occurrence of yeast phase serotypes. The yeast phase cells of H. capsulatum are highly immunogenic (Hill and Marcus, 1959). Mycelial culture filtrates of this fungus also induce a low order

of resistance (Salvin, 1953). It is apparent, then, that there are resistance-inducing immunogens common to both phases.

Marked difference in protective immunogenicity was observed between the saprophytic and parasitic phases of C. immitis. Mycelial elements and arthrospores killed with formalin protected mice against low challenge doses by the intraperitoneal route (Converse, Castleberry, Besemer and Snyder, 1962; Friedman and Smith, 1956; Pappagianis, Miller, Smith, Berman, Kobayashi, 1961). Levine, Cobb, and Smith (1960; 1961) observed that spherules and endospores afforded stronger protection to mice against intranasal challenge doses than did either mycelial elements or arthrospores. Using mycelial elements and spherules grown simultaneously in the same flasks of medium, Kong and Levine (1967) observed that mice vaccinated with spherules were protected against a challenge of approximately 200 LD_{50} compared with approximately 20 LD_{50} in mice vaccinated with mycelial elements.

Employing C. immitis arthrospores, Friedman, Smith and Gordon (1955) studied three strains for comparative virulence in mice. The animals were inoculated intraperitoneally; using three different parameters, the strains showed differing virulence with respect to each other. The authors concluded that virulence is relative and the ideal comparison is made

if the same number of viable particles of each strain has been inoculated into the animals. Inocula containing 100 viable arthrospores per milliliter were used. The order of virulence from the greatest to the least was Silveria, Perry, and Mauser strains.

Using spherules harvested from mice infected with arthrospores Pappagianis, Smith, and Kobayashi (1956) determined the virulence of the *in vivo* form of *C. immitis*. The spherules were ruptured releasing their endospores. The endospores of both strains studied did not show a significant difference in the virulence of endospores and arthrospores.

Levine, Cobb, and Smith (1960) described aspects of protective immunity induced in mice by formalin-killed and purified, mycelial, arthrospore or spherule-endospore vaccines. Intramuscular immunization with 1.6 mg of each preparation resulted in the spherule-endospore vaccine conferring superior protection to that of the mycelial or of the arthrospore preparations, to intranasal challenge doses of 79 to 318 arthrospores. The spherule-endospore preparation was administered in several doses. Better protection was obtained than the same amount given in a single dose.

Pappagianis et al., (1961) immunized mice with viable

C. immitis. Mice vaccinated with dead arthrospores survived intraperitoneal challenge doses but not the intranasal challenge. Mice immunized with viable arthrospores developed a high level of protection surviving both intraperitoneal and intranasal challenge doses.

The culmination of the study on immunity of C. immitis has been the study of the immunogenic properties of non-disrupted and disrupted spherules by Kong, Levine, and Smith (1963). Studies on the cellular fractions demonstrated that the soluble fraction did not afford protection against a challenge dose of 44 arthrospores. The particulate fraction which contained spherules cell walls afforded protection against a challenging dose of 10^2 arthrospores. The recombined fraction showed that the primary immunogenic substance of the spherule was in the cell wall.

The maturation cycle of the tissue phase, peculiar to C. immitis illustrates an additional relationship between morphology and immunogenicity. Levine, Kong, and Smith (1965) showed that immunogenicity increased as the spherule matured and endosporulated. The increase in immunogenicity apparently reflected concomitant biosynthetic developments in the wall, which was shown to contain virtually all the immunogen (Kong, Levine and Smith, 1963).

It seems apparent that induced immunity to fungal

diseases is strongly influenced by the morphological attributes of the vaccine preparation, as well as the routes of vaccination and of challenge. As is evident in Table 1 there has been general agreement that live H. capsulatum is effective in immunizing animals to subsequent challenge. Agreement concerning the efficacy of live vaccines by different investigators does not extend to killed vaccines. Reasons for discrepancies observed with killed vaccines or immunogenic extracts may include variables which become highly critical with killed vaccine or extracts.

Resistance to infection with H. capsulatum can be induced in experimental animals by sublethal infection or by immunization with killed yeast phase cells or polysaccharides derived from these cells. Marcus and Rambo (1952) reported on the determination of LD_{50} values for yeast phase cells injected intravenously and noted differences in virulence of two strains of H. capsulatum by this technique. They also reported that animals infected with a sublethal number of yeast phase cells became resistant to subsequent challenge with lethal numbers of organisms.

Salvin (1953) demonstrated a method for obtaining constant death rates in white Swiss mice. Various routes of inoculation were examined by infecting mice with live cells. Intracerebral inoculation seemed most successful in producing

Table 1

Induced immunity to *Histoplasma capsulatum*:
Summary of findings on vaccination and challenge routes and morphological phase of live and killed preparations

Organism	Live or Killed	Growth Phase	Host	Vaccination Route	Challenge Route	Resistance	Reference
Histoplasma capsulatum	Live	Yeast	Mice	icer*	icer	Strong	Salvin 1955
				ip*	icer	Strong	Salvin 1955
					ip	Increased	Schaefer et al.1954
					iv	Increased	Rambo & Marcus 1955
							Salfelder 1964
				iv*	icer	Strong	Salvin 1955
					iv	Increased	Hill & Marcus 1959
							Rambo & Marcus 1955
							Rowley et al. 1956
				sc*	icer	Strong	Salvin 1955
	Live	Mycelial	Mice	itr*	itr	Increased	Farrell et al. 1953
	Killed	Yeast	Mice	icer	icer	Increased	Salvin 1953
				ip	icer	Strong	Salvin 1953
					ip	Increased	Schaefer et al.1954
					iv	Increased	Hill & Marcus 1959
						No incr.	Rowley & Huber 1956
				im*	iv	Increased	Hill & Marcus 1959
				iv	icer	Strong	Salvin 1953
					iv	Increased	Hill & Marcus 1959
						No incr.	Rowley & Huber 1956
				sc	icer	Strong	Salvin 1953
					iv	Increased	Hill & Marcus 1959
				fp*	icer	Strong	Salvin 1953
				oral	icer	Increased	Salvin 1953
					iv	No incr.	Hill & Marcus 1959

* icer = intracerebral sc = subcutaneous im = intramuscular
ip = intraperitoneal itr = intratracheal fp = foot pad
iv = intravenous

constant death rates. A total of 220 mice were inoculated intracerebrally with yeast phase cells in physiologic saline. The average LD_{50} at 21 days was determined in three separate experiments to be 3.0×10^4 organisms for H. capsulatum strain 6515.

Immunization of mice for short periods of time was accomplished by intraperitoneal inoculation with 5 mg of acetone-dried yeast phase cells, the broth filtrate from the mycelial growth, or the broth filtrate from the growth of the yeast phase cells. Subsequent intracerebral challenge revealed protection against 300-400 LD_{50} of the fungus. Several immunization routes, intraperitoneal, intravenous, and subcutaneous, were effective.

Salvin (1955a) demonstrated that mice immunized with acetone-dried yeast phase cells had less infected tissues (spleen, liver and kidney) than non-immunized mice. The presence of the fungus in tissues was determined by tissue section. The difference between the two groups was most noticeable during the first three weeks after intracerebral challenge with a lethal dose of yeast phase cells. In non-immunized mice very rapid growth of the fungus occurred during the first few hours after challenge, and then a slow and gradual decrease in numbers of yeast phase cells occurred in the tissue cells during the next few months. Immunization

was relative in that it tended merely to lower the number of cells in various tissues.

Resistance to reinfection in experimental histoplasmosis was shown by Salvin (1955b). Mice previously infected with H. capsulatum were found to be more resistant to subsequent intracerebral challenge than normal mice. This resistance was manifested by a decrease in death rate. In mice that developed resistance after a sublethal intraperitoneal inoculation, subsequent intraperitoneal challenge resulted in the greatest number of fungus cells developing in the spleen, liver, and at the site of injection. In intracerebrally challenged mice which were immunized by a sublethal intracerebral injection, the fungus cells occurred in the liver, spleen, and brain. In control animals after intracerebral challenge, the organisms were found in lungs and kidney. In general, previous infection tended to inhibit the growth of the fungus in the spleen, liver, and at the site of inoculation.

Rowley and Huber (1956) studied the growth of H. capsulatum in normal, "superinfected" (rechallenge of infected animals), and immunized mice. Chronic infections were produced in mice by injections of small numbers of viable yeast phase cells, approximately 70 aggregates. These mice were resistant to the lethal effects of 10^6 aggregates

injected intravenously. Growth of the fungus was inhibited in superinfected mice whereas in controls, after initial rapid multiplication, there was a progressive decline in the number of viable organisms in the tissues. Mice immunized with a killed yeast phase cell vaccine, 10^9 cells given intraperitoneally or 10^8 cells given intravenously, failed to show any effect on the growth of H. capsulatum in tissues. It was apparent that immunization with killed yeast phase cells did not inhibit growth of the fungus in the liver and spleen after intravenous challenge of 80 cells.

Survival of H. capsulatum in experimental histoplasmosis was demonstrated by Saslaw and Schaefer (1956). Mice were immunized and challenged by the intraperitoneal route of injection. The fungus was recovered frequently from the reticuloendothelial tissues as late as 45 weeks after infection. Mice surviving previous sublethal challenge or those previously given one dose of heat-killed organisms showed little variation. The only difference observed was a greater number of positive organ cultures in the sublethal group between 16 and 30 weeks.

Hill and Marcus (1959) studied the quantitative aspects of resistance induced against H. capsulatum. Using the intravenous route, LD_{50} determinations were studied using the yeast phase of the fungus. Strains exhibiting low and

high virulence were found. Groups of mice were immunized by intraperitoneal injection of formalin-killed yeast cells of each strain. The animals were challenged intravenously with varying numbers of yeast phase cells of the high virulence strain. Either strain of the fungus was as effective as the other in immunization when compared to non-immunized control animals. The effect of route of immunization using either subcutaneous, intramuscular, intraperitoneal or intravenous routes was studied. It was found that similar resistance levels were achieved by each route. Infection-immunity was studied for its effect upon subsequent challenge. The group that was sublethally infected showed the same type of dose-dependent mortality relationship that was observed in the other groups of immunized animals.

An important consideration was the dose of vaccine. The extent of immunity induced by killed yeast phase H. capsulatum was found to be dose-dependent (Schaefer and Saslaw, 1954; Salvin, 1955) and at high doses the extent of resistance was comparable to that following sublethal infection (Saslaw and Schaefer, 1954; Salvin, 1955b; Hill and Marcus, 1959). Using live organisms the immune response to H. capsulatum was found to be dose-dependent (Salvin, 1955a). Although the dependency was less pronounced than with killed organisms, the presence of the immune response

demonstrated that strong stimulation occurred only after a threshold of immunogens was attained. Speculations concerning the high immunogenicity of live organisms have been discussed by Salvin (1960). One speculation is that the live organisms multiply and thereby increase their immunogen content in the host. Such multiplication was evident after sublethal infection with Histoplasma (Rambo and Marcus, 1954; Hill and Marcus, 1959).

Resistance evoked by live organisms was little influenced by the parenteral immunization route used (Table 1). The vaccination route appeared to be of major importance in fungal immunity induced by nonliving vaccines. Aside from variations in the virulence of fungus strains (Drouhet and Schwarz, 1956; Howell and Kipkie, 1950) it is well documented that the capacity of a given strain to kill animals varies with the route of infection and with the host. The LD_{50} of H. capsulatum for mice increases, in terms of numbers of organisms, when administered in the following order: intracerebrally, intravenously, intraperitoneally (Salvin, 1955a). However, using the intramuscular, intraperitoneal, subcutaneous, and intravenous routes for immunization, Marcus and Hill (1959) found similar resistance achieved by each immunization route to subsequent intravenous challenge. In monkeys (Macaca mulatta) intratrachael and intravenous injections of

H. capsulatum yeast phase appeared to produce a more severe disease than that following intranasal inoculation (Saslaw, Carlisle and Sparks, 1960). In mice the intranasal route generally caused more severe histoplasmosis (Salvin, 1955a) than the intraperitoneal route. Immunized mice showed a lowered resistance to respiratory challenge than to challenges by other routes (Grayston and Salvin, 1956). These workers found that mice given killed yeast cells of H. capsulatum showed less severe pathological changes after intracerebral challenge than after intranasal challenge.

The foregoing reports indicate the importance of the challenge route in vaccine studies. The intracerebral, intravenous, and intraperitoneal routes generally allow the organisms to disseminate rapidly to various organs, whereas the respiratory route, in appropriate doses, initiates primarily a pulmonary disease (Grayston and Altman, 1954; Procknow, Page, and Loosli, 1960) with extra-pulmonary dissemination as a later occurrence.

It seems apparent from the reports concerning H. capsulatum that immunity induced by live organisms persists longer than that induced by killed organisms. The duration of immunity evoked by live organisms may be attributed to prolonged stimulation provided by the persistence of the fungus in the host tissue (Salvin, 1955a). Salvin (1955b) found that

resistance to histoplasmosis induced by a live vaccine was first detected when the mice were challenged three days after immunization and that it remained strong for at least 14 days. Immunity following sublethal infection was also effective against H. capsulatum (Schaefer and Saslaw, 1954) at 40 days and at 90 days (Hill and Marcus, 1959).

However, with killed vaccines, immunity to histoplasmosis declines over a relatively short period. Using a vaccine from yeast phase of H. capsulatum, Salvin (1955b) reported a decline with time, in resistance to intracerebral challenge. Mice were protected against a challenge of 320 LD_{50} administrated six to fourteen days postvaccination but at 24 days they were protected only against 10 LD_{50}.

IV. MANIFESTATION OF THE IMMUNE RESPONSE IN HISTOPLASMOSIS

The most stringent tests of efficacy in experimental immunization are the prevention of overt infection after challenge or the induction in the host of resistance sufficient to clear infection organisms without the development of serious illness. Live yeast phase (Salvin, 1955b) preparations have enabled animals to withstand severe challenge and to survive for extended periods.

Suppressed growth of H. capsulatum has also been reported in mice immunized with live or killed organisms and challenged intranasally, intraperitoneally, intravenously,

or intracerebrally, and in guinea pigs challenged intraperitoneally (Salvin, 1955a; 1955b). Initially after challenge, multiplication was most pronounced at the site of infection in both control and immunized mice, and the extent of brain and kidney involvement varied with the route of infection (Salvin, 1955b). Growth of H. capsulatum occurs largely within cells of the reticuloendothelial system and the organisms or lesions containing the organisms have been found invariably in the liver and spleen regardless of the challenge route (Grayston and Altman, 1954; Grayston and Salvin, 1956; Larsh and Cozad, 1965; Procknow, Page, and Loosli, 1960; Salvin, 1965). Rowley and Huber (1956) indicated that growth of the organisms in tissues was inhibited in intravenously challenged mice only by the use of live cell vaccines. The six-day post challenge interval employed by Rowley might have been too short to detect low levels of inhibition.

In the study by Grayston and Salvin (1956) brain involvement in control mice was extensive after intracerebral challenge, but there were only minimal inflammatory infiltrates in mice vaccinated with live or killed organisms. Differences in the liver were less marked but granulomas were few and well organized in immunized mice. The spleens in both groups contained many organisms but few lesions.

The killed cell vaccine appeared to be less effective against intranasal challenge than against intracerebral challenge. Pathological involvement after intranasal challenge was limited and lung and liver lesions in the control group were resolved spontaneously.

Delayed dermal hypersensitivity reaction to H. capsulatum has been elicited in infected guinea pigs (Johnson and Scherago, 1960; Knight and Marcus, 1958; Marcus, Aoki, and Hill, 1965), rabbits (Markowitz, 1964), monkeys (Saslaw, Carlisle, and Sparks, 1960) and rats (Okudaira and Schwartz, 1962). Killed organisms or their fractions also were effective in sensitizing guinea pigs (Larsh, 1960; Salvin, 1955a).

Because of the relative difficulty in skin testing in the mouse, delayed hypersensitivity in this animal was tested by injecting histoplasmin either intravenously or into the foot pad. Early deaths in mice previously vaccinated with live or killed H. capsulatum were produced either by intravenous injection of organism (Box and Briggs, 1961) or by intraperitoneal injection of organisms in mucin (Salvin, 1958). The state of hypersensitivity persisted for 14 months (Box and Briggs, 1961).

V. IMMUNOGENS OF HISTOPLASMA CAPSULATUM

The antigens which are protective in H. capsulatum have been found in the cell wall. The yeast phase walls were as

immunogenic as killed whole cells and the protoplasmic fraction contained virtually no activity (Salvin and Ribi, 1955). The cell wall of the dimorphic fungus contains lipids, polysaccharides, proteins, and chitin-like substances (Blank, 1954; Pine, Boone, and McLaughlin, 1966). It is presumed to be the locus of protective and serologically active antigens in the yeast phase of the organism.

Salvin and Smith (1959) isolated a protein-carbohydrate complex from culture filtrates of autolyzed yeast phase Histoplasma cells and it was as immunogenic as killed cells. In contrast, the protective capacity of a predominantly polysaccharide-containing preparation used by Knight, Hill and Marcus (1959) was inferior to that of killed cells. The two findings were not necessarily contradictory; neither the isolation and purification procedures were the same nor were the immunization and challenge regimens similar.

The specificity of immunity induced by H. capsulatum has been examined by cross challenge both with heterologous organisms having antigens in common and with those not so constituted. Live yeast phase H. capsulatum increased resistance against challenge with Blastomyces dermatitidis (Salfelder and Schwartz, 1964) in mice. Killed yeast cells increased resistance to Candida albicans challenge (Hasenclever and Mitchell, 1963). Hedgecock (1961) found that

immunization with a vaccine prepared from the mycelial phase of H. capsulatum increased resistance to challenge by Mycobacterium tuberculosis. Induced immunity is not strain-specific in experimental histoplasmosis (Hill and Marcus, 1959; Rambo, Marcus, and Gunn, 1955).

VI. MECHANISMS OF THE IMMUNE RESPONSE TO HISTOPLASMA CAPSULATUM

Mechanisms and development of immunity to the mycoses have received little study. The significance of antihistoplasma antibodies in resistance is still obscure. Such antibodies conferred no passive protection on recipient mice (Rowley and Huber, 1956; Salvin, 1960). The importance of a cellular role in immunity to H. capsulatum was demonstrated by accelerated infiltration of inflammatory cells and associated development of granulomatous lesions (Grayston and Salvin, 1956).

Concerning the role of phagocytosis in immunity with H. capsulatum, an intracellular organism, contradictory results have been reported. In one study, the extent of fungal growth in macrophages from vaccinated mice was the same as that in macrophages from control mice (Howard, 1965). In other studies, (Hill and Marcus, 1960) prior immunization increased the digestive capacity of macrophages (Miya and Marcus, 1961; Wu and Marcus, 1963).

Studies in animals designed to elucidate the patho-

genesis of H. capsulatum infection and to develop potent vaccines have been hampered by intrinsic and extrinsic factors. The hazards involved in handling this pathogenic spore-forming fungus, and its morphological variability present difficulties in experimentation. Nevertheless, inroads into the understanding of fungal immunity have been made; enhanced resistance to the fungus has been achieved by immunization. Also the site of the immunogenic substance(s) appears to have been localized in the yeast phase cell wall. Immunomycology has received less attention than viral and bacterial immunology. However, as epidemiological and ecological knowledge expands, public health statistics point increasingly to the desirability of fungal vaccines, especially in regions of endemic systemic mycotic disease.

MATERIALS AND METHODS

I. ORGANISMS

Cultures of H. capsulatum in the yeast phase were maintained at 37° C on antibiotic human blood agar. These were transferred at weekly intervals.

Mycelial cultures of H. capsulatum were maintained at room temperature on Sabouraud's dextrose agar and were transferred every three to six months. Strains were also maintained under sterile mineral oil.

Table 2 lists the twenty-three strains of H. capsulatum used in these studies and indicates the origin of the culture. These strains were originally isolated from human and animal sources, and from soil samples.

The yeast phase of the strains of the fungus were obtained by in vitro or in vivo conversion of mycelial elements. In vitro conversions were obtained by transferring mycelial elements onto blood agar slants containing antibiotics and after incubation at 37° C. Close-growing moist mycelial colonies developed which were transferred until the yeast phase appeared. Mice were injected either intraperitoneally or intravenously with a saline suspension of the mycelial elements to obtain in vivo conversion. The mice were autopsied at the end of two and four weeks and specimens

Table 2

Origin of stock cultures of *Histoplasma capsulatum*

Strain number	Origin	Strain number	Origin
2171[a]	human, undated	3072[a]	human, undated
2247	human, undated	3154	from India, 1965
2584	human, 1957	3289	human, 1965
2585	soil, 1957	3321	human, 1965
2586	human, 1954	3330	human, 1965
2645	human, 1957	G10[b]	human, 1949
2779	from Japan, 1957	G56	var. *duboisi*, undated
2813	human, undated	G72	human, 1959
2870	mouse, undated	G75	human, 1960
2888	human, undated	6651[c]	human, undated
3014	soil, 1960	G17M[d]	human, 1955
3021	opossum, 1960		

[a] obtained from Dr. N. F. Conant, Duke University
[b] obtained from Dr. C. Campbell, Harvard University
[c] obtained from Dr. C. Emmons, NIH
[d] obtained from University of Utah stock culture collection

from liver, lung, and spleen inoculated onto antibiotic blood agar plates. The resulting yeast phase organisms were then transferred onto blood agar slants.

II. MICE

Adult albino mice (Mus musculus) of mixed sexes, obtained from local sources were used in all experiments. Mice were maintained on Purina Laboratory Chow or Rockland mouse diet.

III. MEDIA

All cultures were grown and maintained on one of the following media:

Liquid. Yeast phase organisms for vaccine production were grown in the following liquid culture medium (Hill, 1958).

Tryptose phosphate broth (Difco)	29.4 g
Yeast extract	4.0 g
Maltose	10.0 g
Cystine	0.5 mg
Distilled water	1000 ml

Solid. The medium used for in vitro conversion, maintenance and plate counts was antibiotic blood agar (Hill and Marcus, 1959).

Tryptose phosphate broth (Difco)	29.5 g
Agar (Difco)	20.0 g
Human blood	15-20%
Distilled water	800 ml

A final concentration of 25-50 units of penicillin and 25-50 μg of streptomycin per ml of medium were added.

The medium used for maintenance of the stock culture was Sabouraud's dextrose agar (Ajello, Georg, Kaplan, and Kaufman, 1963).

Dextrose	40 g
Peptone	10 g
Agar	20 g
Distilled water	1000 ml

Final pH of 5.6.

Five media were employed in studying the sporulation characteristics and colony appearance.

Modified Sauton's medium (Willis and Cummings, 1952).

Dextrose	50.0 g
Asparagine	4.0 g
Citric acid	2.0 g
Glycerin	50.0 g
$KH_2PO_4 \cdot 3H_2O$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NH_4 ferric citrate	0.05g
Agar	20 g
Distilled water	1000 ml

Final pH of 7.4.

Sabouraud's medium with phosphate (McVeigh and Morton, 1965).

Dextrose	40.0 g
Peptone	10.0 g
KH_2PO_4	1.5 g
Agar	20 g
Distilled water	1000 ml

Final pH of 5.6.

The following three synthetic media were modified after Artis and Baum (1963).

Synthetic Medium One

Dextrose	10.0 g
KH_2PO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.15g
$(NH_4)_2SO_4$	2.0 g
Agar	20.0 g
Distilled water	1000 ml

Synthetic Medium Two

Medium two was identical to one except 2 g of asparagine was substituted for the $(NH_4)_2SO_4$.

Synthetic Medium Three

Medium three was identical to medium one except for the $(NH_4)_2SO_4$ which was excluded.

A final pH after sterilization of 7.2 was obtained in these synthetic media.

IV. LD_{50} DETERMINATIONS

Animals were injected intravenously with 0.5 ml of the yeast cell suspensions. The organisms used were grown at 37° C upon antibiotic blood agar. The inoculum was prepared by suspending the organisms in 1% tryptose phosphate broth (Difco) in 0.9% NaCl (PSS). After aseptically filtering through glass wool, total numbers of cells were estimated from counts made in a hemocytometer. Aggregates of yeast phase cells were counted as one. The concentration of the yeast phase organisms was adjusted with 1% tryptose phosphate broth so that each dose of organism was injected intravenously in 0.5 ml volume. The mortality ratio results after 30 days were analyzed by the use of either the method of Miller and Tainter (1944) or the method of Litchfield and Wilcoxon (1949).

V. STRAIN DESCRIPTION

Twenty-three strains of *H. capsulatum* maintained on Sabouraud's dextrose agar in the mycelial phase were described as to their macroscopic appearance. The media employed were: modified Sauton's medium, Sabouraud's medium containing phosphate ion, and synthetic media 1, 2, and 3. The nitrogen source was asparagine in Sauton's medium, peptone in

Sabouraud's medium, and $(NH_4)_2SO_4$ in synthetic medium 1, asparagine in synthetic 2, and no added nitrogen in synthetic medium 3.

Plates were poured from each agar medium and inoculated with mycelial elements. The plates were sealed with parafilm and incubated at room temperature. The plates were observed frequently for contamination as well as type of growth. After 10 weeks of growth the cultures were killed by exposure to formaldehyde fumes for 2-7 days at room temperature. This procedure killed all cells as demonstrated by failure to multiply on Sabouraud's agar. The macroscopic appearance of the colonies was described in mycological nomenclature and documented with photographs.

VI. SPORE MEASUREMENTS

Microscope slides were made of each strain grown on each medium. The preparations were observed for the production of microconidial and macroconidial spore types. The method of preparation of the microscope slides was based on a procedure described by Dr. N. F. Conant (personal communication). A piece of transparent "sticky tape" was cut to fit under a coverslip. The sticky tape was then pressed against the formalin-killed growth of the fungus. The tape was removed with the spores and hyphal elements

adhering and placed sticky side up onto a microscope slide. Lacto-phenol cotton blue was dropped onto the tape and the coverslip applied. The preparation was pressed with the eraser end of a pencil to remove any trapped air bubbles from beneath the coverslip.

Lacto-phenol cotton blue mounting fluid was prepared according to the following formulation: (Ajello, Georg, Kaplan, and Kaufman, 1963)

Phenol crystals	20.0 g
Lactic acid	20.0 g
Glycerin	40.0 g
Cotton blue (Poirrier's blue)	0.05g
Distilled water	20.0 ml

The diameters of the microconidia and of the macroconidia were measured using an ocular micrometer. The diameter was measured from cell wall surface to surface not including the tuberculations of either spore type. The ocular micrometer was standardized for the optics of the microscope in use in this laboratory using a stage micrometer. Spore sizes are averages of six to ten measurements.

The relative numbers of the spores were determined. A numerical rating was employed as follows: none observed (0), rare (1), few (2), and numerous (3). "None observed" is self explanatory. The slides were observed for two to

five minutes using the low power objective (total magnification, 60X) for macroconidia, and then the high dry objective (total magnification, 264X) for macroconidia. "Rare" means the spores were difficult to find; only by moving to various oil immersion fields (total magnification, 600X) were any observed. "Few" is defined as observing one field in which one or two spores could be found. "Numerous" is defined as more than two spores per observed oil immersion field.

Using this rating system, a number was calculated to represent macroconidial and microconidial spore production in the various media. A ratio of macroconidia to microconidia was determined for each strain on the five media.

VII. VACCINE PREPARATION

Strains of H. capsulatum were grown in the mycelial phase of Sabouraud's dextrose agar containing phsophate ion. After 10 weeks of growth the cultures were killed by exposure to formaldehyde fumes as described previously. The mycelial growth was removed aseptically by scraping the colony surface with a scalpel after covering the surface of the large plastic petri dishes (150 x 15 mm) with saline containing 0.1% Tween 80. The resulting suspension, composed of spores and mycelial elements, was concentrated by centrifugation

(800 X g) and then washed in saline solution. After the final wash, the spores and mycelial elements were suspended in saline.

Yeast phase suspensions of the organisms were grown in liquid culture medium. One hundred ml of medium was placed in 500 ml Erlenmeyer flasks. Suspensions of yeast phase organisms grown on antibiotic blood agar were used as inocula. The cultures were incubated at 37° C until maximum growth was obtained (3-7 days). The flasks were agitated by a mechanical platform shaker (16 rpm) during the incubation period. Formalin was added to a final concentration of 0.5% and the flasks were kept at 37° C overnight. This procedure killed all cells as demonstrated by culture on antibiotic blood agar. The cells were concentrated by centrifugation (800 X g) and then washed in saline. After the final wash the cells were suspended in saline.

After the final wash, the suspension of yeast phase or mycelial phase cells in saline was transferred to a 450 ml solution bottle (3" x 7" diameter, height) containing numerous glass marbles (16 mm diameter) and stainless steel balls (10 mm and 6 mm diameters). The bottle was then placed upon revolving rollers. The ball mill was allowed to run continuously for 48 hours (mycelial phase) or 168 hours (yeast phase) in the refrigerator (ca. 5° C). After

grinding the cellular debris was removed by centrifugation at low speed (400-500 X g) and the supernate fluid employed as vaccine (designated a cell free extract).

Macroconidia were separated from the mycelium and microconidia using a floation method (Stewart and Meyer, 1932). The spores and hyphal elements were removed after covering the surface of the petri dish with sterile distilled water. The surface was scraped lightly with a scalpel. The suspension was removed and pipetted into a large glass tube (7 3/4" x 1 1/4"). The upper layer was removed after a 20 minute settling period. The procedure was repeated twice to further remove the mycelium and microconidia from macroconidia.

The vaccine preparations were weighed by pipetting 1.0 ml of each preparation onto tared steel planchets. Three aliquots of each vaccine preparation were dried in an oven at 87° C until constant weight was reached. After the dry weights were obtained, appropriate dilutions were made for immunization procedures.

VIII. TESTS USED TO CHARACTERIZE VACCINES

The vaccine preparations were tested for nitrogen and total hexose concentration. The methods were found in Kabat and Mayer (1962) and will only be summarized.

Estimation of protein with Folin-Ciocalteu phenol reagent

The method used is a modification for the estimation of 10-100 ug of nitrogen. The error reported for repeated determinations is about ± 1 ug nitrogen.

Reagents:

1. Folin reagent (Fisher Scientific Co.).
2. 12.5% solution of Na_2CO_3 (anhydrous).
3. 0.1% solution of $CuSO_4 \cdot 5H_2O$.

Procedure:

Two ml of solution were measured into a tube. Six ml of the Na_2CO_3 solution and 1.0 ml of the $CuSO_4$ solution were added, mixed and allowed to stand for one hour at room temperature. One ml of 1:3 freshly diluted Folin reagent was slowly added with constant mixing. After 20 to 30 minutes, the determinations (standard solution and unknown solutions) were read at 750 mu against a blank of 2 ml of distilled water to which all the reagents had been added. The Coleman Model 30 spectrophotometer was used.

Anthrone reaction for total hexoses

The reaction depended upon the formation of furfural derivatives and was best used in the range of carbohydrate from 50-250 ug.

Reagent:

1. Two grams anthrone in one liter of concentrated H_2SO_4.

Procedure:

Ten ml of anthrone reagent were pipetted into test tubes in a water bath at 10-15° C. The sample (5 ml) was carefully layered above the reagent. Blanks and standards of glucose were included. Each tube was agitated until mixed. The tubes were warmed to room temperature and heated at 90° C for 16 minutes, cooled and read in the Coleman Model 30 spectrophotometer at 625 mu.

IX. IMMUNIZATION

Groups of mice were immunized by using the prepared formalin-killed vaccines. Each animal received an intraperitoneal injection of 0.25 ml using various schedules as noted in the experiment.

EXPERIMENTAL RESULTS

I. MACROSCOPIC APPEARANCE OF COLONIES

Description of each strain of H. capsulatum on five different media grown at room temperature for 10 weeks is presented in detail.

Each strain was described according to the following: 1) Sabouraud's dextrose medium with phosphate ion, (Table 3); 2) modified Sauton's medium, (Table 4); 3) modified Sauton's medium without dextrose, (Table 5); 4) synthetic medium 1, (Table 6); and 5) synthetic medium 2, (Table 7). The macroscopic colonial characteristics were not described for synthetic media 3. Growth was scant on this medium which contained no added nitrogen source.

Growth on synthetic medium 1 was similar to that obtained in synthetic medium 2. Sabouraud's and Sauton's media both grew colonies differing in appearance. The nitrogen source in each medium was: 1) Sabouraud's, peptone; 2) Sauton's, asparagine; 3) synthetic 1, $(NH_4)_2SO_4$; and 4) synthetic 2, asparagine. The carbohydrate source in all of the media was dextrose.

Sabouraud's and Sauton's media supported best growth in these experiments. Synthetic 2 also supported good growth. On synthetic 1, synthetic 3 and Sauton's without

Table 3

Strains of *Histoplasma capsulatum* grown on Sabouraud's dextrose medium with phosphate ion for ten weeks

Strain G-10:	colony flat, granular, with tan and white concentric rings.
Strain G17M:	colony uniformly tan with concentric rings and lobate edges.
Strain G-56:	colony cottony white and tan concentric rings.
Strain G-72:	colony cottony, flat, tan with white variation.
Strain G-75:	colony white and cottony, flat.
Strain 2171:	colony variegated white and tan, flat and cottony.
Strain 2247:	entire colony tan, wooly, with concentric rings.
Strain 2584:	colony center raised and folded, surface wooly, central color light tan, concentric ring of darker brown.
Strain 2585:	colony tan with powdery surface raised from agar surface and folded with numerous radial grooves.
Strain 2586:	entire colony heaped, glabrous ridges and powdery valleys.
Strain 2645:	colony flat, powdery, with cottony white peripheral growth and tan to brown center.
Strain 2779:	colony surface smooth and wooly with few folds, gray to tan in color.

Table 3 (continued)

Strain 2813:	colony center heaped, central area gray, edges tan to brown.
Strain 2870:	colony center heaped with irregular folds, entire colony powdery and tan.
Strain 2888:	colony cottony with heaped center, center gray, peripheral growth light tan.
Strain 3014:	colony heaped and irregular folds, surface powdery, peripheral growth tan.
Strain 3021:	colony dark tan, center raised and cottony, outside margin powdery.
Strain 3072:	colony center heaped, periphery with irregular folds, color of center white to tan with edges tan and powdery.
Strain 3154:	colony gray with brown concentric rings, center heaped with many cracking ridges.
Strain 3289:	colony divided, one gray and cottony with ridges, the other tan and powdery with heaped and ridged areas.
Strain 3321:	entire colony heaped with irregular folds, colony uniformly gray and powdery.
Strain 3330:	colony center crateriform, edges with irregular folds, folds powdery white, colony center glabrous.
Strain 6651:	colony with white cottony center, peripheral growth white to tan, entire colony flat.

Table 4

Strains of *Histoplasma capsulatum* grown on modified Sauton's medium for ten weeks

Strain G-10:	colony with white powdery peripheral growth, surface at the center is glabrous, waxy with radial folds and steel-gray in color.
Strain G17M:	colony crateriform with steel-gray center which is waxy and glabrous, peripheral growth granular to cottony.
Strain G-56:	colony heaped with a white fluffy surface.
Strain G-72:	colony white and powdery, center heaped and crateriform.
Strain G-75:	no growth.
Strain 2171:	colony with raised center with radial folds, color even throughout, surface tan and granular.
Strain 2247:	colony heaped and irregularly folded, surface powdery to downy, center glabrous and steel-gray with color following some of the radial grooves.
Strain 2584:	colony center cottony, concentric rings of gray, outside ring white, with margin powdery and gray.
Strain 2585:	colony center raised and purple with peripheral growth gray with powdery appearance.
Strain 2586:	colony glabrous, center raised, color light brown.
Strain 2645:	colony powdery and granular with steel-gray glabrous center.

Table 4 (continued)

Strain 2779:	colony uniformly white, center raised with radiating ridges.
Strain 2813:	colony variegated brown and white, center raised, peripheral growth with dark brown concentric ring.
Strain 2870:	colony center grayish black with ridges, peripheral growth with brown concentric ring.
Strain 2888:	colony center heaped with ridges, dark gray in color, peripheral growth light gray, colony uniformly wooly.
Strain 3014:	colony center raised with numerous ridges, peripheral growth powdery and brown.
Strain 3021:	colony center raised and dark brown, outside margin gray and powdery.
Strain 3154:	colony center heaped and glabrous, dark brown in color, peripherally colony white radially furrowed and wooly.
Strain 3321:	colony center raised with many radial ridges, colony white to light gray except for concentric ring which is dark gray.
Strain 3330:	colony center brown and raised, edges powdery and white.
Strain 6651:	colony flat with folds, peripheral growth powdery to cottony, center raised, steel-gray and waxy.

Table 5

Strains of *Histoplasma capsulatum* grown on modified Sauton's medium without dextrose for ten weeks

Strain G17M:	colony uniformly gray and wooly.
Strain G-75:	colony uniformly dark tan and powdery.
Strain 2171:	colony white with raised center.
Strain 2584:	colony uniformly tan and cottony.
Strain 2645:	colony center raised and light tan, peripheral growth brown.
Strain 2870:	colony grayish white and wooly.
Strain 2888:	colony dark brown with light tan concentric ring.
Strain 3321:	colony center raised and dark gray, peripheral growth grayish white.
Strain 3289:	colony center raised with ridges, color grayish white.

Table 6

Strains of *Histoplasma capsulatum* grown on Synthetic medium 1 for ten weeks

Strain G-10:	colony center slightly raised, entire colony glabrous and smooth, colony cream colored.
Strain G17M:	colony center raised, surface cottony, color creamy.
Strain G-56:	colony center elevated, surface cottony and white.
Strain G-72:	colony center heaped and cracked, surface wooly and cream colored.
Strain G-75:	colony white, raised center.
Strain 2171:	colony gray, entire colony shallowly wrinkled.
Strain 2247:	colony tan, entire colony deeply wrinkled.
Strain 2584:	colony center raised, entire colony glabrous and dark tan.
Strain 2585:	growth scant, tan and crusty.
Strain 2586:	colony white with tan raised central area, crescent shaped tan surface area peripheral, remainder of colony wooly.
Strain 2645:	colony glabrous and smooth, tan in color, center raised.
Strain 2779:	colony light tan, center raised, uniformly powdery.

Table 6 (continued)

Strain 2813:	colony center raised and light tan, colony cottony to powdery and light tan.
Strain 2870:	colony white with raised center, cottony.
Strain 2888:	colony center raised, glabrous, and gray, peripheral growth wooly and white.
Strain 3014:	colony center crateriform, edges lobed, entire colony powdery and tan.
Strain 3021:	colony center heaped, periphery wooly and light tan.
Strain 3072:	colony center slightly raised, entire colony wooly and light tan.
Strain 3154:	colony center heaped, entire surface powdery and dark tan.
Strain 3289:	colony glabrous and crusty, entire surface tan.
Strain 3321:	colony center heaped, uniformly gray and powdery.
Strain 3330:	colony center raised, entirely white and powdery.
Strain 6651:	colony whitish, raised center, glabrous margin.

Table 7

Strains of *Histoplasma capsulatum* grown on

Synthetic medium 2 for ten weeks

Strain G-10:	colony center white raised with furrows, peripheral growth scant, tan, and adherent.
Strain G17M:	colony center crateriform with radial furrows, growth white, peripheral growth tan and adherent.
Strain G-56:	colony white with patches of tan, peripheral growth scant, cottony.
Strain G-72:	colony whitish with diffuse tan throughout, raised center, radial grooves near periphery, growth generally granular.
Strain G-75:	colony uniformly white, with raised center, growth wooly.
Strain 2171:	colony variegated white and tan, center raised and furrowed.
Strain 2247:	colony center gray-white, raised and furrowed, peripheral growth white and cottony.
Strain 2584:	colony uniformly grayish white, smooth and powdery.
Strain 2585:	colony center heaped, and folds few and irregular, entire colony white and cottony.
Strain 2586:	colony center crateriform, edges glabrous, center powdery white, edges smooth and tan.
Strain 2645:	colony center crateriform, edges smooth and powdery, central colony dark tan, periphery lighter.

Table 7 (continued)

Strain 2779: colony tan with white concentric ring, uniformly powdery.

Strain 2813: colony center heaped, entire colony smooth, powdery and light tan.

Strain 2870: colony cottony, smooth and white.

Strain 2888: colony with white central region with wide peripheral, band of center powdery, band glabrous.

Strain 3014: colony gray with light tan center, cottony and raised from agar surface.

Strain 3021: colony center heaped, and glistening, irregular folds to edges, entire colony white.

Strain 3072: colony center heaped, powdery and white, peripheral growth tan and wooly.

Strain 3154: colony center raised slightly, entire colony smooth and cottony, colony center light tan, edges dark tan.

Strain 3289: entire colony dark tan, smooth and powdery.

Strain 3321: colony center slightly heaped, central colony light tan, edges tan and wooly.

Strain 3330: colony center raised with few folds, center tan and powdery, edges white and cottony.

Strain 6651: colony flat entirely white, with scant growth in peripheral areas.

dextrose the strains of H. capsulatum grew poorly and after 10 weeks, only covered the central area of the petri dishes.

Figure 2 shows color photographs of macroscopic colonies of four strains of H. capsulatum grown at room temperature for 10 weeks on Sabouraud's medium with phosphate ion. The strains shown are: G10, G17M, G56, and 2779.

II. SPORE SIZE MEASUREMENTS

Spore size measurements were made from observations on 23 strains of H. capsulatum grown on Sabouraud's dextrose media with phosphate ion, modified Sauton's medium, and synthetic media 1, 2, and 3 (Table 8). Both macroconidia and microconidia were measured. The values were reported as averages for both of the spore types. The blanks in the table represent cultures which failed to grow or were contaminated. The zeros indicate that no spores were found of that type.

The macroconidial and microconidial spores ranged in size from 6.5 - 14.3 u and 2.3 - 5.8 u. The averages of the measured spores were: 1) Sabouraud's macroconidia, 12.2 u and microconidia, 3.8 u; 2) Sauton's, 10.5 u and 4.0 u; 3) synthetic 1, 9.4 u and 3.8 u; 4) synthetic 2, 9.7 u and 3.2 u; and finally 5) synthetic 3, 10.9 u and 3.5 u. The largest macroconidia (12.2 u) were formed on Sabouraud's

Strain G10

Strain G17M

Strain G56

Strain 2779

Figure 2. Four strains of Histoplasma capsulatum grown on Sabouraud's dextrose medium with phosphate ion at room temperature for ten weeks.

Table 8

Histoplasma capsulatum macroconidia and microconidia spore sizes on five media

Strain No.	SAB c̄ PO_4	Sauton's	#1	#2 Synthetic	#3
G10	--*	--	9.8/0	10.9/0	9.5/0
G17M	--	0/2.7	7.3/3.0	9.0/3.0	0/2.8
G56	13.1/5.7**	10.1/3.7	11.6/5.2	10.4/3.6	9.9/3.5
G72	--	0/0	9.7/4.0	0/3.2	0/3.0
G75	0/0	--	0/3.5	--	--
2171	--	--	0/0	--	0/2.7
2247	0/3.0	--	9.3/2.3	11.6/3.1	10.6/3.2
2586	--	0/0	0/4.7	0/2.3	0/0
2585	11.8/0	10.0/4.2	9.1/4.3	0/3.5	8.3/3.3
2585	0/0	0/0	0/3.7	0/0	0/0
2645	14.3/4.5	--	8.9/3.8	8.5/4.9	12.6/5.7
2779	10.7/3.1	12.8/0	12.0/4.2	9.3/3.0	9.0/4.0
2813	12.1/4.0	10.5/5.8	9.8/2.7	9.1/2.9	11.7/4.5
2870	11.0/0	0/0	0/0	0/0	11.0/4.3
2888	0/0	0/0	9.1/3.8	0/0	0/0
3014	0/3.5	0/0	10.4/5.0	0/0	12.0/0
3021	12.0/3.0	10.3/4.5	9.3/4.3	0/0	14.2/3.8
3072	13.5/5.7	9.5/4.0	10.5/4.9	0/3.5	9.7/2.9
3154	10.2/0	12.5/3.8	10.5/3.7	11.0/3.1	10.0/3.3
3289	0/2.5	10.0/3.3	9.0/2.7	9.5/3.3	13.6/3.8
3321	13.7/3.1	8.5/0	6.5/2.9	9.3/2.7	0/3.3
3330	0/3.8	11.1/0	0/3.7	0/3.0	0/2.7
6651	--	--	7.0/4.5	8.5/2.8	0/3.2

* (data not obtained)
** macroconidia/microconidia, diameter in microns

with phosphate ion. The largest microconidia were formed on Sauton's (4.0 μ). Mean sizes for microconidia on Sabouraud's with phosphate and synthetic 1 were both 3.8 μ.

The results shown in Figures 3, 4, and 5 were the average and the range of each spore size for each strain represented. Each point represents the macroconidial and microconidial spore size for each strain with the macroconidial size increasing along the abscissa. The results of spore size measurements of synthetic media 1, 2, and 3 showed that no correlation existed between the size of the macroconidia and microconidia on synthetic media.

III. RELATIVE NUMBERS OF SPORES

Table 9 shows the relative numbers of macroconidia based on the rank system previously described. For each strain grown on each of the five media, the rank ratios given were for macroconidia over microconidia. Discarded cultures were indicated by blanks.

The last column in the table shows a number which represented the sporulation character of the strains of H. capsulatum studied. A number above 1.00 indicated that the strain had produced large numbers of macroconidia, a number below 1.00 indicated that the microconidia predominated. A number of 1.00 indicated that the strain produced an equal

Figure 3. Histoplasma capsulatum macroconidial and microconidial spore size on synthetic medium 1.

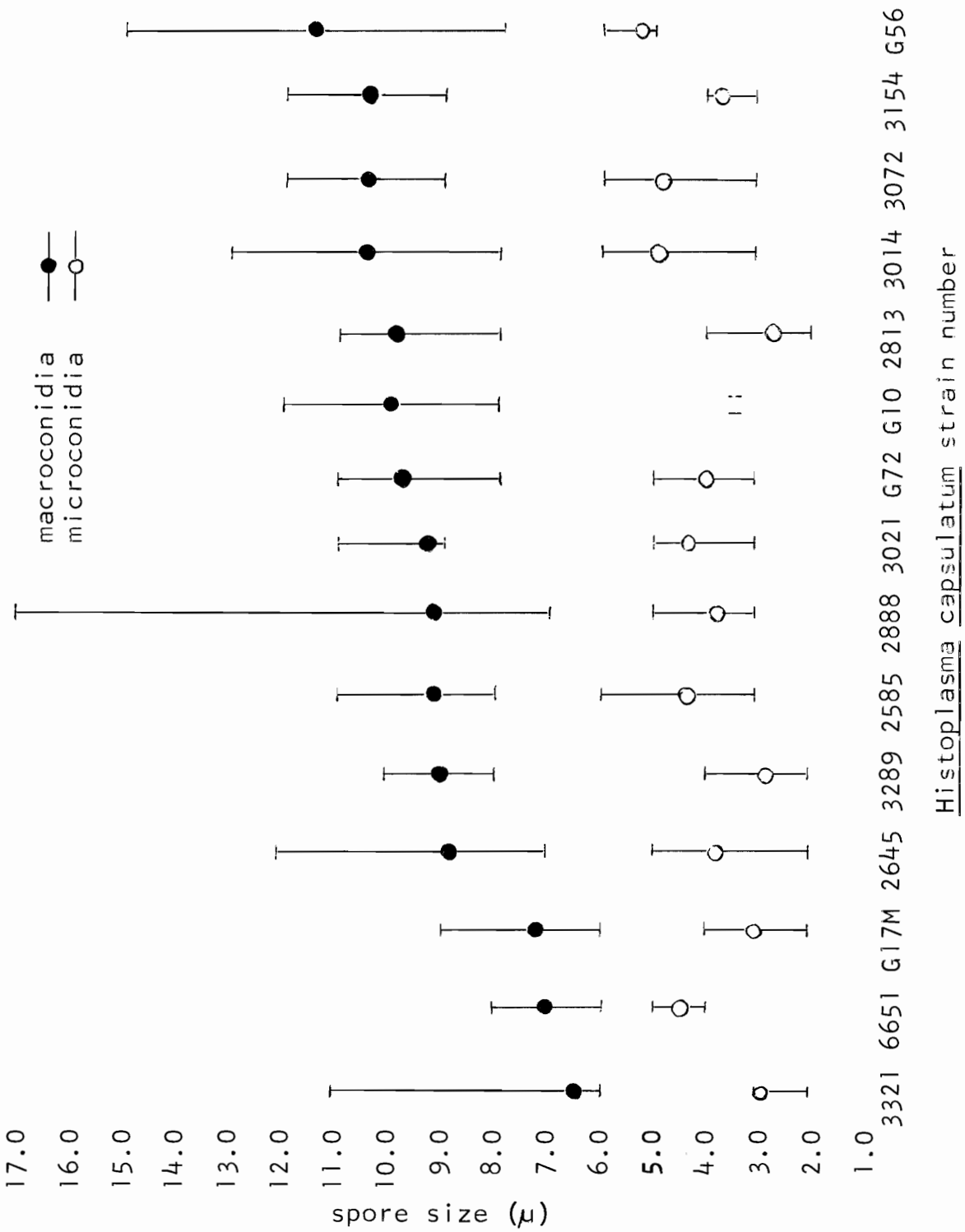

Figure 4. Histoplasma capsulatum macroconidial and microconidial spore size on synthetic medium 2.

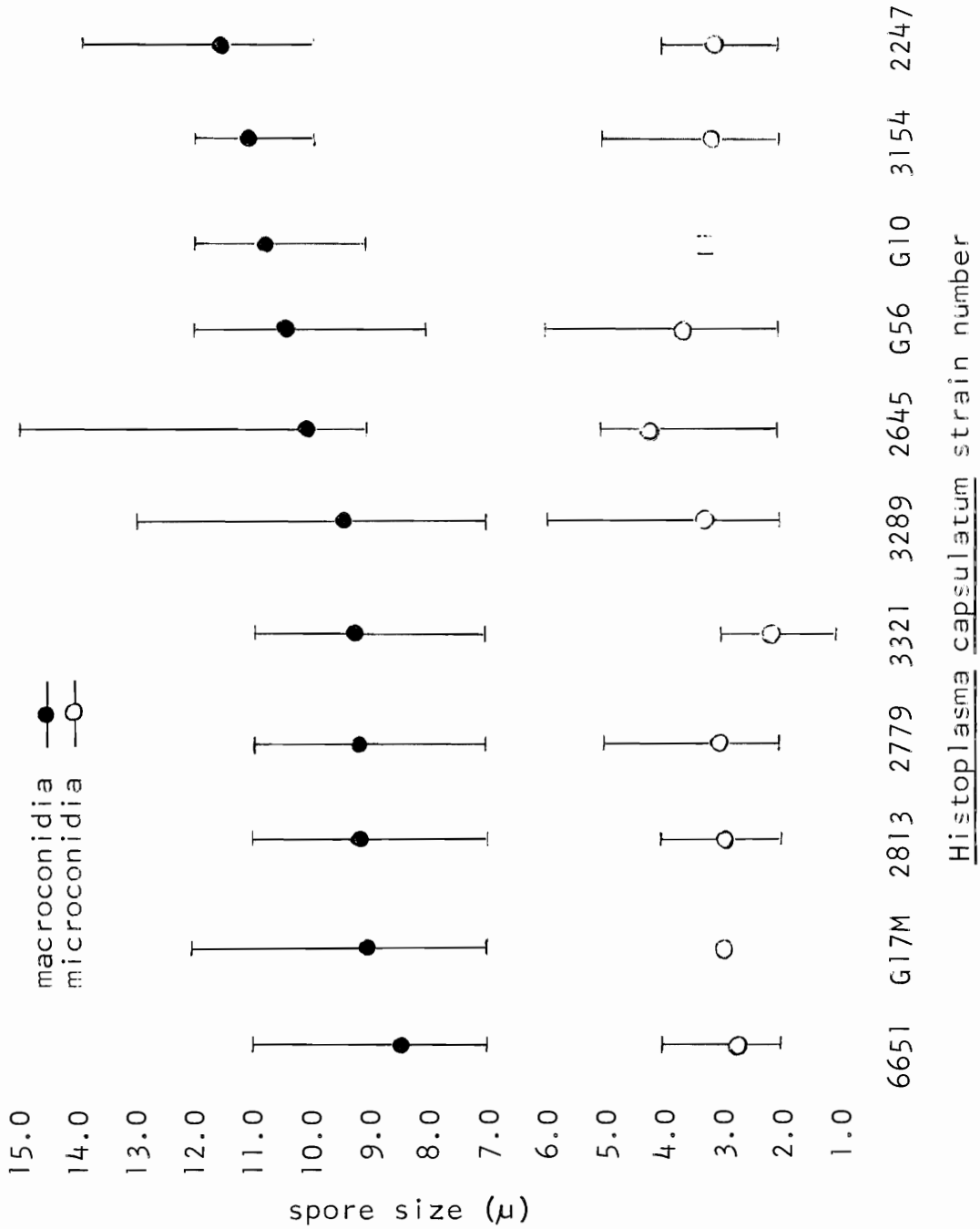

Figure 5. Histoplasma capsulatum macroconidial and microconidial spore size on synthetic medium 3.

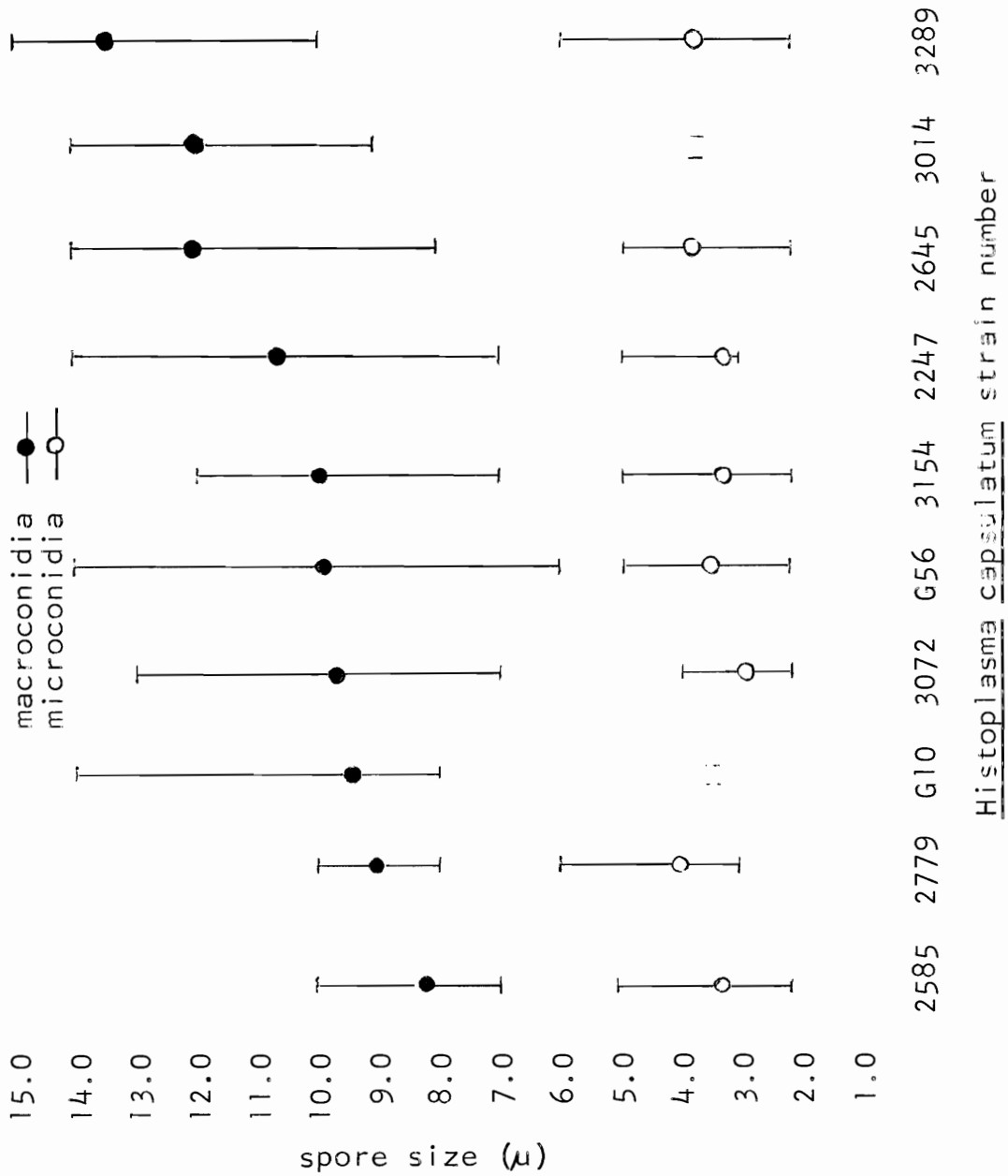

Table 9

Histoplasma capsulatum relative numbers of macroconidia and microconidia on five media

Strain No.	SAB c̄ PO_4	Sauton's	#1	#2	#3 Synthetic media	Sporulation number*
G10	3/3[a]	3/3	2/0	3/0	1/0	2.00
G17M	2/2	0/3	3/2	2/1	2/1	1.00
G56	3/3	3/3	3/2	3/2	3/2	1.25
G72	---	0/0	2/2	0/3	0/1	0.33
G75	0/0	---	0/1	0/0	0/0	0.00
2171	2/0	2/3	0/0	0/0	0/2	0.80
2247	0/3	0/2	2/2	3/3	3/3	0.62
2584	---	0/0	0/1	0/2	0/0	0.00
2585	3/0	2/2	1/2	0/3	1/2	0.78
2586	0/0	0/0	0/1	0/0	0/0	0.00
2645	3/2	3/2	2/2	3/3	3/2	1.27
2779	2/2	2/0	1/2	3/2	2/2	1.25
2813	3/2	3/2	3/3	2/3	3/2	1.17
2870	1/0	0/0	0/0	0/0	1/1	2.00
2888	0/0	0/0	3/3	0/0	0/0	1.00
3014	0/1	0/0	1/2	0/0	1/0	0.67
3021	2/2	3/2	1/2	0/0	3/2	1.13
3072	3/1	2/1	2/3	0/1	3/3	1.11
3154	1/0	3/1	3/3	3/3	1/1	1.38
3289	0/3	3/1	2/3	3/2	3/1	1.10
3321	3/2	2/0	2/3	1/3	0/2	0.80
3330	0/1	1/0	0/2	0/1	0/1	0.20
6651	2/3	2/3	1/2	3/2	0/2	0.67

[a] macroconidia rank number/microconidia rank number
* relative numbers of macroconidia to microconidia

number of macroconidia and microconidia. The figures for strain G10 served as an example for the calculations of the spore number:

	SAB + PO_4	Sauton	Syn. 1	Syn. 2	Syn. 3
G10	3/3	3/3	2/0	3/0	1/0

For the macroconidial number each numerator was added: 3 + 3 + 2 + 3 + 1 = 12. Similar calculations determined the number for the microconidia, i.e. a denominator of six. The ratio, sum of the numerators over sum of the denominators, i.e., 12/6 or 2.00 was the sporulation number and was a relative measure of the type of spores produced by the strain. The value did not give any indication of the abundance of spores produced.

The data presented suggested that a correlation existed between nutritional properties of the media employed and sporulation ability. This is based on the following observations: two of 23 strains (G10 and 2870) produced a predominance of macroconidia as indicated by the sporulation number. However, the number of spores produced by G10 was abundant on four of the five media, whereas strain 2870 produced only scant numbers of spores on two of the five media. Two of 23 strains (G75 and 2586) produced only microconidia. Synthetic medium 1 yielded low numbers of microconidia from strains G75 and 2586, but no sporulation

occurred on the remainder of the media examined. Two of 23 strains (G17M and 2888) produced an equal number of both spore types as indicated by the sporulation number. On Sauton's medium G17M did not produce macroconidia and on the remaining media the microconidia and macroconidia were produced in varying numbers. Both (G17M and 2888) of these strains produced varying numbers or no spores on the media on which they were grown. Two strains (G72 and 3330) produced a greater number of microconidia than macroconidia, as indicated by the sporulation number. The remaining strains yielded sporulation numbers that varied between the two extremes (0.20-2.00).

Although the sporulation number characterized the relation between total macroconidia and microconidia formed, it gave no estimate of the abundance of spores formed. For example, strain G10 formed only macroconidia on the synthetic media but the number of macroconidia formed falls into the classification "rare." On the other hand this strain forms equal and large numbers of both spore types on Sabouraud's medium with phosphate.

Calculations were made similar to those described above but designed in this case to describe sporulation for each medium. The rank numbers of each strain on each medium were added, i.e. adding vertically in Table 9. Media which

supported best macroconidial or microconidial production could be determined using this method. Results are recorded in Table 10.

The results suggested that the medium which supported the best macroconidial sporulation was Sabouraud's with phosphate. Synthetic media 1 and 2, and Sauton's medium supported better microconidial production. Synthetic medium 3 supported formation of equal numbers of the two spore types.

IV. VIRULENCE STUDIES

In order to study the resistance of mice to infection with yeast phase H. capsulatum, it was desirable to know the virulence of selected strains and the effect that prolonged maintenance on artificial culture media had upon this virulence. Therefore, LD_{50} determinations were performed at various times throughout the course of this study. Data are presented in Table 11 which were obtained from LD_{50} determinations of H. capsulatum strain G17M, at different periods. Data in the table prior to 1956 were taken from Hill (1958). The virulence of this strain of H. capsulatum did not change significantly over a period of 15 years. Furthermore, other factors such as mouse passage, human passage, and conversion did not change the virulence of this strain.

Table 10

Media supporting the best sporulation of

Histoplasma capsulatum

Medium	Sporulation number
Sabouraud's with phosphate ion	1.30
Modified Sauton's	0.83
Synthetic medium 1	0.79
Synthetic medium 2	0.88
Synthetic medium 3	1.00

Table 11

Mouse LD_{50} determinations of yeast phase

Histoplasma capsulatum strain G17M

Strain	Date	LD50*	
G17 a)	12/17/53	1.1×10^6	$(0.76 - 1.6 \times 10^6)$+
G17 a)	5/11/54	2.7×10^6	$(1.0 - 7.0 \times 10^6)$
G17-5 b)	10/18/54	9.0×10^5	$(0.35 - 2.3 \times 10^6)$
G17-10 b)	11/04/54	7.5×10^5	$(0.28 - 2.0 \times 10^6)$
G17-15 b)	1/31/55	1.2×10^6	$(0.66 - 2.2 \times 10^6)$
G17 M c)	4/21/55	6.5×10^5	$(0.33 - 1.3 \times 10^6)$
G17 M c)	8/19/56	1.2×10^6	$(0.80 - 1.8 \times 10^6)$
G17 M a)	6/20/66	5.0×10^5	$(0.02 - 9.8 \times 10^5)$
G17 M a)	7/08/66	1.1×10^6	$(0.92 - 1.3 \times 10^6)$
G17 M a)	7/10/66	6.4×10^5	$(3.4 - 6.4 \times 10^6)$
G17 M a)	5/19/68	7.0×10^5	$(0.18 - 1.2 \times 10^6)$

* LD_{50} determined by method of Miller and Tainter (1944)

\+ 95% confidence limits

a) conversion from mycelial phase

b) mouse passage, one/week for 15 weeks; determination at 5, 10, and 15 weeks

c) after human passage

Strain 3154 of H. capsulatum was found to be of the same virulence for mice over an 18 month period. Table 12 presents data obtained from LD_{50} determinations with this strain.

Table 13 presents data obtained from virulence determinations on various strains of H. capsulatum at different times throughout this study. The virulence of three of these strains (2645, 3330, and 6651) appeared to be similar. Strain 2813 was the least virulent of the six strains that were studied.

V. IMMUNIZATION

Using the sporulation number as a basis for determining the morphological characteristics of a strain, four strains were chosen to represent the mycelial phase of H. capsulatum, (Table 14). Strain G17M was used as the source of yeast phase cells.

Mycelial phase cultures were grown on Sabouraud's dextrose agar for 10 weeks at room temperature and harvested. The harvest from the cultures was placed in a ball mill and a cell free extract was prepared. The cell free extract was preserved with formalin and used as a vaccine. A whole macroconidial vaccine prepared with spores from strain G10 was also used to immunize mice.

Table 12

Mouse LD_{50} determinations of yeast phase

Histoplasma capsulatum strain 3154

Strain	Date	LD_{50}*	
3154	March, 1967	1.4×10^4	$\pm$(88, 900)+
3154	October, 1967	9.6×10^5	$\pm$(60, 000)
3154	January, 1968	6.0×10^4	$\pm$(82, 000)
3154	April, 1968	3.4×10^5	$\pm$(160, 000)
3154	June, 1968	6.2×10^5	$\pm$(170, 000)
3154	May, 1968	3.0×10^5	$\pm$(200, 000)

* LD_{50} and standard deviation determined by method of Miller and Tainter (1944)

\+ one standard deviation

Table 13

Mouse LD_{50} determinations of yeast phase *Histoplasma capsulatum* of various strains

Strain	Date		
2645	June, 1966	2.4×10^5	$\pm(2.0 \times 10^5)$
2645	December, 1967	7.6×10^5	$\pm(2.9 \times 10^5)$
2813	March, 1967	3.0×10^8	$\pm(4.3 \times 10^8)$
3330	April, 1968	7.0×10^5	$\pm(4.5 \times 10^5)$
6651	October, 1965	5.0×10^5	$\pm(9.7 \times 10^5)$

* lethal dose 50 ± standard deviation after Miller and Tainter (1944)

Table 14

Strains of *Histoplasma capsulatum* utilized in immunity studies

Strain	Element produced	Sporulation number
G10	Macroconidia	2.00
G75	Mycelium	0.00
3330	Microconidia	0.20
2645	Mixture of both spores	1.27

Yeast phase cultures were grown on liquid medium and harvested after five days at 37° C. The cells were either pulverized in a ball mill or were left intact and utilized as a vaccine.

All groups of mice in these reported experiments were immunized by intraperitoneal injection of either formalin-killed cell free extracts or formalin-killed whole cells. The doses of the challenging strain were also similar in each experiment, i.e., 1×10^5, 1×10^6, and 1×10^7 population units injected intravenously.

After termination of each experiment the remaining mice were autopsied and the lungs were cultured on antibiotic blood agar.

The data were analysed by the method of Litchfield and Wilcoxon (1949) and the LD_{50} and potency ratios are given in each experiment.

In the first experiment (Table 15) the cell free extract prepared from strain G10 was used. The sporulation number for this strain of H. capsulatum indicated that the strain produced mainly macroconidia. Mice in one group received a 3 mg (dry weight) injection of the vaccine. Mice in the second group were injected with 2 mg. The third group of mice were injected with 1 mg. The control group was injected with saline (PSS). Ten days after the initial

Table 15

Vaccination of mice against histoplasmosis

Mice vaccinated with formalin-killed cell free extract of the mycelium and macroconidia of G10 and challenged with 3154

challenge dose	30 day mortality ratios, per cent deaths, and culture results							
	PSS control		3 mg		2 mg		1 mg	
1×10^5	1/9 (6/8	11% [a] 75%)[b]	0/10 (1/10	0 10%)	0/10 (1/10	0 10%)	0/11 (2/11	0 18%)
1×10^6	8/10 (2/2	80% 100%)	0/10 (1/10	0 10%)	0/10 (2/10	0 20%)	1/10 (3/9	10% 33%)
1×10^7	10/10 (0/0	100% 0	3/10 (4/7	30% 57%)	0/10 (6/10	0 60%)	1/10 (5/9	10% 55%)
Totals	19/29 (8/10	66% 80%)	3/30 (6/27	10% 22%)	0/30 (9/30	0 30%)	2/31 (10/29	7% 35%)
LD_{50}[c]	$4.2 \times 10^5 \pm (2.5 \times 10^5)$		$1.3 \times 10^7 \pm (1.2 \times 10^7)$		———		$1.0 \times 10^6 \pm (9.4 \times 10^6)$	
P.R.[d]	———		31 (10-95)		———		24 (7-83)	

a mortality ratio and per cent deaths

b culture positive lungs/total survivors

c LD_{50} = lethal dose 50 and standard deviation

d potency ratio and 95 per cent confidence limits

injections all mice were challenged intravenously with strain 3154.

The results of immunization with G10, macroconidial vaccine are shown in Table 15 and suggest that there was a difference between 3 mg and 1 mg in one injection. The per cent deaths and culture results indicated no difference between the two groups immunized with either 2 mg or 1 mg. A lower frequency of culture positive lungs occurred in the group immunized with 3 mg. The potency ratio comparison between 3 mg and 1 mg was 31 and 24 respectively.

The results in a second experiment involving immunization with G10 and challenge after 10 days with 3154 are shown in Table 16. The culture results indicated that as the dose of the challenge was increased the number of culture positive lungs increased. Although the total culture positive percentage was similar in the 0.8 mg and 0.4 mg groups, the potency ratio for 0.4 mg was 18.5 and the ratio for 0.1 mg was 5.0.

Results in two experiments involving mice immunized with a cell free extract of the mycelium of G75 (a strain producing no spores) are recorded in Tables 17 and 18. The saline controls for both experiments have equal LD_{50} values of 1.3×10^6. The number of culture positive lungs for yeast phase growth, varied from 12% in the 0.8 mg group to

Table 16

Vaccination of mice against histoplasmosis

Mice vaccinated with formalin-killed cell free extract of the mycelium and macroconidia of G10 and challenged with 3145

30 day mortality ratios, per cent deaths, and culture results

challenge dose	PSS control	3 mg	2 mg	1 mg
1×10^5	1/8 13% [a] (3/7 43%)[b]	not done	0/8 0% (2/8 25%)	1/9 11% (6/8 67%)
1×10^6	3/8 38% (4/5 90%)	not done	1/9 11% (6/8 67%)	3/8 38% (4/5 90%)
1×10^7	8/8 100% (0/0 0)	1/5 20% (3/5 60%)	4/9 45% (4/5 90%)	6/10 60% (4/4 100%)
Totals	12/24 50% (7/12 58%)	(1/5 20% (3/5 60%)	5/26 38% (12/21 57%)	10/27 37% (14/17 38%)
LD_{50}[c]	$7.0 \times 10^5 \pm (4.0 \times 10^5)$	——	$1.3 \times 10^7 \pm (1.6 \times 10^7)$	$3.5 \times 10^6 \pm (7.2 \times 10^6)$
P.R.[d]	——	——	18.5 (6-57)	5.0 (1.6-15)

a mortality ratio and per cent deaths

b culture positive lungs/total survivors

c LD_{50} = lethal dose 50 and standard deviation

d potency ratio and 95 per cent confidence limits

Table 17

Vaccination of mice against histoplasmosis

Mice vaccinated with formalin-killed cell free extract of the mycelium of G75 and challenged with 3154

challenge dose	30 day mortality ratios, per cent deaths, and culture results			
	PSS control	3 mg	2 mg	1 mg
1×10^5	0/10 0%[a] (7/10 70%)[b]	1/9 11% (0/8 0%)	0/8 0% (1/8 13%)	1/7 14% (2/6 33%)
1×10^6	7/10 70% (3/3 100%)	1/9 11% (1/8 13%)	0/8 0% (0/8 0)	0/7 0% (3/7 43%)
1×10^7	8/10 80% (2/2 100%)	1/9 11% (5/8 63%)	1/7 14% (4/6 67%)	0/7 0% (6/7 86%)
Totals	15/30 50% (12/15 80%)	3/27 11% (6/24 25%)	1/23 4% (5/22 23%)	1/21 5% (11/20 55%)
LD_{50}[c]	$1.3 \times 10^6 \pm (1.1 \times 10^6)$	———	$2.6 \times 10^7 \pm (1.6 \times 10^7)$	———
P.R.[d]	———	———	20 (7.6-53)	———

a mortality ratio and per cent deaths

b culture positive lungs/total survivors

c LD_{50} = lethal dose 50 and standard deviation

d potency ratio and 95 per cent confidence limits

Table 18

Vaccination of mice against histoplasmosis

Mice vaccinated with formalin-killed cell free extract of the mycelium of G75 and challenged with 3154

30 day mortality ratios, per cent deaths, and culture results

challenge dose	PSS control	0.8 mg	0.4 mg	0.1 mg
1×10^5	1/10 10% [a] (8/9 89%) [b]	2/10 20% (0/8 0%)	0/7 0% (2/7 29%)	0/8 0% (1/8 13%)
1×10^6	7/10 70% (2/3 67%)	0/11 0% (0/11 9%)	1/7 14% (2/6 33%)	1/8 13% (4/7 57%)
1×10^7	7/10 70% (3/3 100%)	0/7 0% (2/7 29%)	6/10 60% (4/4 100%)	4/10 40% (3/6 50%)
Totals	15/30 50% (13/15 87%)	2/28 7% (3/26 12%)	7/24 29% (8/17 47%)	5/26 19% (8/21 38%)
LD_{50} [c]	$1.3 \times 10^6 \pm (1.8 \times 10^6)$	——	$6.1 \times 10^6 \pm (6.5 \times 10^6)$	$1.3 \times 10^7 \pm (1.7 \times 10^7)$
P.R. [d]	——	——	4.7 (1.3-18)	10 (2.3-43)

a mortality ratio and per cent deaths

b culture positive lungs/total survivors

c LD_{50} = lethal dose 50 and standard deviation

d potency ratio and 95 per cent confidence limits

80% in the saline control group. The potency ratios are as follows: 2 mg, 20; 0.4 mg, 4.7; and 0.1 mg, 10. The injection of 2 mg of vaccine gave the highest protection based on the potency ratio.

The cell free extract vaccine prepared from strain 3330, was used to immunize mice and yielded the results shown in Table 19. The potency ratios were: 1 mg, 11.8; 0.4 mg, 7.6 and 0.1 mg, 1.5. The culture results indicated that as the dose of the vaccine was increased the number of culture positive lungs decreased.

Using the cell free extract of strain 2645, which yielded a mixture of both types of spores, as a vaccine the potency ratios were: 15.4 for 3 mg and 9.3 for 2 mg and 1 mg. The results are shown in Table 20. The total per cent culture positive lungs for each group were: controls, 42%; 3 mg, 23%; 2 mg, 14%; and 1 mg, 31%.

Vaccination of mice with the cell free extract prepared from the yeast phase of strain G17M gave results shown in Table 21. The potency ratios are: for 0.4 mg a value of 23 and for 0.1 mg a value of 11.5. The culture result totals ranged from 11-92%.

Mice immunized with whole cells of either macroconidia of strain G10 or yeast cells of G17M gave the results shown in Table 22. The potency ratio calculated for each group of

Table 19

Vaccination of mice against histoplasmosis

Mice vaccinated with formalin-killed cell free extract of the mycelium and microconidia of 3330 and challenged with 3154

30 day mortality ratios, per cent deaths, and culture results

challenge dose	PSS control	2 mg	1 mg	0.4 mg	0.1 mg
1×10^5	not done	not done	0/7 0 [a] (2/7 29%) [b]	1/7 14% (1/6 17%)	2/7 29% (2/5 40%)
1×10^6	5/7 72% (2/2 100%)	0/5 0% (2/5 40%)	1/7 14% (2/6 33%)	2/8 25% (2/6 33%)	3/8 38% (3/5 60%)
1×10^7	7/7 100% (0/0 0%)	0/5 0% (1/5 20%)	2/9 22% (4/7 57%)	4/8 50% (3/4 75%)	7/8 88% (1/1 100%)
Totals	12/14 86% (2/2 100%)	0/10 0% (3/10 30%)	3/23 12% (3/20 40%)	7/23 30% (6/16 37%)	12/23 52% (6/11 55%)
LD_{50} [c]	$4.6 \times 10^5 \pm (2.9 \times 10^5)$	——	$5.4 \times 10^6 \pm (5.5 \times 10^6)$	$3.5 \times 10^6 \pm (1.3 \times 10^6)$	$7.0 \times 10^5 \pm (1.2 \times 10^6)$
P.R. [d]	——	——	11.8 (4.2-31)	7.6 (NC)	1.5 (0.43-5.2)

a mortality ratio and per cent deaths

b culture positive lungs/total survivors

c LD_{50} = lethal dose 50 and standard deviation

d potency ratio and 95 per cent confidence limits

Table 20

Vaccination of mice against histoplasmosis

Mice vaccinated with formalin-killed cell free extract of mycelium and spores of 2645 and challenged with 3154

challenge dose	30 day mortality ratios, per cent deaths, and culture results			
	PSS control	3 mg	2 mg	1 mg
1×10^5	2/10 20% [a] (2/8 25%)[b]	0/8 0% (0/8 0)	0/10 0% (1/10 10%)	0/9 0% (0/9 0%)
1×10^6	7/10 70% (2/3 67%)	0/9 0% (3/9 33%)	0/9 0% (0/9 0%)	0/9 0% (3/9 33%)
1×10^7	9/10 90% (1/1 100%)	5/9 55% (2/5 40%)	7/10 70% (2/3 67%)	7/10 70% (3/3 100%)
Totals	18/30 60% (5/12 42%)	5/26 21% (5/22 23%)	7/29 24% (3/22 14%)	7/28 25% (6/21 31%)
LD_{50}[c]	5.6×10^5 $\pm(5.9 \times 10^5)$	8.6×10^6 $\pm(6.2 \times 10^6)$	5.2×10^6 $\pm(3.2 \times 10^6)$	5.2×10^6 $\pm(3.2 \times 10^6)$
P.R.[d]	———	15.4 (4.7-50)	9.3 (2.9-29)	9.3 (2.9-29)

a mortality ratio and per cent deaths

b culture positive lungs/total survivors

c LD_{50}= lethal dose 50 and standard deviation

d potency ratio and 95 per cent confidence limits

Table 21

Vaccination of mice against histoplasmosis

Mice vaccinated with formalin-killed cell free extract of yeast phase G17M and challenged with 3154

challenge dose	30 day mortality ratios, per cent deaths, and culture results			
	PSS control	1 mg	0.4 mg	0.1 mg
1×10^5	1/8 13% [a] (7/7 100%)[b]	0/9 0% (0/9 0%)	0/8 0% (0/8 0%)	0/9 0% (1/9 11%)
1×10^6	3/8 38% (4/5 90%)	0/8 0% (1/8 13%)	0/8 0% (2/8 25%)	1/8 13% (2/7 28%)
1×10^7	8/8 100% (0/0 0%)	0/8 0 (2/8 25%)	1/8 13% (6/8 67%)	3/8 38% (3/5 60%)
Totals	12/24 50% (11/12 92%)	0/27 0% (3/27 11%)	1/24 47% (3/23 34%)	4/25 16% (6/21 28%)
LD_{50}[c]	$7 \times 10^5 \pm (4.4 \times 10^5)$	——	$1.6 \times 10^7 \pm (1.4 \times 10^7)$	$8.0 \times 10^6 \pm (9.4 \times 10^6)$
P.R.[d]	——	——	23 (7.8-69)	11.5 (3.6-38)

a mortality ratio and per cent deaths

b culture positive lungs/total survivors

c LD_{50} = lethal dose 50 and standard deviation

d potency ratio and 95 per cent confidence limits

Table 22

Vaccination of mice against histoplasmosis

Mice vaccinated with formalin-killed whole spores or yeast phase cells and challenged with 3154

30 day mortality ratios, per cent deaths, and culture results

challenge dose	PSS control	G10 Macroconidia 7.3×10^4	G17M whole cells	
			10^6 cells	5×10^7 cells
1×10^5	2/10 20% [a] (6/8 67%)[b]	0/7 0 (5/7 71%)	0/9 0 (4/9 45%)	0/8 0 (4/8 50%)
1×10^6	5/10 50% (5/5 100%)	3/8 38% (3/5 60%)	4/9 45% (3/5 60%)	1/7 14% (2/6 33%)
1×10^7	9/10 90% (1/1 100%)	6/8 75% (2/2 100%)	3/9 33% (5/6 84%)	2/10 20% (6/8 75%)
Totals	16/30 53% (12/14 86%)	9/23 39% (10/14 72%)	7/27 25% (12/20 60%)	3/25 12% (12/22 55%)
LD_{50}[c]	$7.0 \times 10^5 \pm (1.7 \times 10^6)$	$2.4 \times 10^6 \pm (2.1 \times 10^6)$	$5.0 \times 10^6 \pm (4.9 \times 10^6)$	$8.0 \times 10^6 \pm (8.9 \times 10^6)$
P.R.[d]	———	3.4 (0.9-12.5)	7.2 (2-28)	11.4 (2.6-51)

a mortality ratio and per cent deaths

b culture positive lungs/total survivors

c LD_{50} = lethal dose 50 and standard deviation

d potency ratio and 95 per cent confidence limits

immunized mice were: G10 (7.3×10^4 cells), 3.4; G17M (1×10^6 cells), 7.2; and G17M (5×10^7 cells), 11.4. The culture results were similar: G10 macroconidia, 72%; G17M (1×10^6), 60%; and G17M (5×10^7), 55%. Eighty-six per cent of the saline controls were culture positive. Results recorded in Table 23 show a summary of the potency ratios for the vaccines prepared from the various strains of H. capsulatum. The results indicated that cell free extracts either of strain G10 (mycelial phase) or of strain G17M (yeast phase) had similar potency ratios. The ratios are: G10 - 0.4 mg, 18.5; 0.1 mg, 5.0 and G17M - 0.4 mg, 23; 0.1 mg, 11.5, for identical vaccine doses. Strains G75, 2645, and 3330 cell free extract vaccines had potency ratios which varied from 1.5 to 20.0. The whole cell vaccines prepared from strains G17M and G10 had ratios between 3.4 and 11.4.

Determinations of the chemical composition of the vaccines prepared from certain H. capsulatum strains are shown in Table 24. Strains G10 and G17M have similar carbohydrate-protein ratios of 57 and 56 respectively. The remaining vaccines had ratios that varied between 6.7 and 12.

Table 23

Comparison and summary of potency ratios of vaccines from various strains of *Histoplasma capsulatum*

Strain	Amount[a] Injected	Potency[b] Ratio	Description
G10	3.0 mg	31.0 (10-95)	mycelial phase CFE [c]
	1.0 mg	24.0 (7-83)	
	0.4 mg	18.5 (6-57)	
	0.1 mg	5.0 (1.6-15)	
G75	2.0 mg	20.0 (7.6-53)	mycelial phase CFE
	0.4 mg	4.7 (1.3-18)	
	0.1 mg	10.0 (2.3-43)	
2645	3.0 mg	15.4 (4.7-50)	mycelial phase CFE
	2.0 mg	9.3 (2.9-29)	
	1.0 mg	9.3 (2.9-29)	
3330	1.0 mg	11.8 (4.2-31)	mycelial phase CFE
	0.4 mg	7.6 (NC) [d]	
	0.1 mg	1.5 (0.43-5.2)	
G17M	0.4 mg	23.0 (7.8-69)	yeast phase CFE
	0.1 mg	11.5 (3.6-38)	
G17M	10^6 (0.53 mg)	7.2 (2-28)	formalinized yeast cells
	5×10^7 (2.7 mg)	11.4 (2.6-51)	
G10	7.3×10^4	3.4 (0.9-12.5)	formalinized macroconidia

[a] dry weight of suspension injected

[b] potency ratio and 95% confidence limits determined by Litchfield and Wilcoxon (1949)

[c] CFE (cell free extract)

[d] not calculated

Table 24

Chemical composition of five strains of

Histoplasma capsulatum

Strain	Vaccine Description	%CHO[a]	%Protein[b]	Ratio[c]	Dry Weight
G10	mycelial phase, CFE*	34%	0.6%	57.0	23.8 mg
G75	mycelial phase, CFE	47%	7.1%	6.7	6.3 mg
G17M	yeast phase, CFE	39%	0.7%	56.0	9.4 mg
2645	mycelial phase, CFE	35%	2.9%	12.0	10.9 mg
3330	mycelial phase, CFE	33%	4.4%	7.5	17.1 mg
G17M	yeast phase, whole cells	39%	3.2%	9.1	10.5 mg

* CFE (cell free extract)

a) carbohydrate (CHO) determined by anthrone method

b) protein determined by Folin and Ciocalteu method

c) ratio % CHO/% protein

DISCUSSION

H. capsulatum is composed of a thallus which may be considered as both vegetative and reproductive. The vegetative thallus is composed entirely of mycelial filaments of hypha, which are generally branched and spread in all directions from which the spores arise. The reproductive part includes all structures which lead to the formation and dispersal of the various spore types.

The extreme polymorphism of fungi is a major challenge to students of mycology. Doctors, veterinary surgeons, and clinical laboratory and public health workers familiar with the life cycles of protozoa and helminths which are parasitic on man and animals will appreciate the significance of the range of fungal forms.

Morphological reduction in size associated with parasitism may be regarded as a particular case of fungal polymorphism (Langeron and Vanbrueseghen, 1965). Thus, the fungal parasite, *H. capsulatum*, has a saprophytic stage composed of varied forms and a parasitic state which is yeast-like.

The occurrence of histoplasmosis in man and animals is related to the colonization of suitable natural substrates in the environment by histoplasma. Apparently, histoplasmosis is contracted by inhalation of spores after exposure

to the fungus growing in the environment. H. capsulatum has been isolated from this environment which includes soil and debris from: chicken houses, caves, hollow trees, barnyards, river bottoms including water, human and animal tissues. Available laboratory specimens, identified as H. capsulatum by observing typical macroconidia, have been given various strain designations by individuals who isolated the fungus. The designations range from letters and numbers to the name of the patient or locale of the isolate. Identification is currently based solely on morphology. This situation leads to some strange results. First, available isolates must always be identified as H. capsulatum after transfer to a new laboratory. This can be a tedious procedure when a relatively asporogenous strain is being investigated (eg., G75). The current arbitrary designation of strains may be based on locale of isolation, in which case it is not clear whether the organism was isolated from soil, a lesion (human or animal) or fomites. Again, strain designation may be based on whether the organism was isolated from a particular area. For example, one strain used in this study, 3154, is labeled "India." The origin of the strain is again unclear.

The large number of strains each laboaratory maintains results in much cumbersome duplication of effort. It must

be granted that little is known about H. capsulatum, and that some means for identifying strains of the fungus in terms of origin as well as species, must exist. The procedures used in the identification of pathogenic bacteria might well be followed. The classification of pathogenic bacteria falls in the following general groupings: 1) morphology and staining, 2) cultural characteristics such as fermentation and pigment production, 3) serology including antigenic structure, and 4) production of a disease process.

A keystone in the classification of higher plants and animals is the definition of a species as a group of individuals capable of continued fertile interbreeding (Davis et al., 1968). However, this keystone does not apply to bacteria and fungi for which the situation is quite different. The number of characteristics available which enable species identification for bacteria and fungi is limited. There is no known example of ontogeny to recapitulate phylogeny; there is no fossil record; and finally, reproduction is ordinarily asexual. The definition of Davis, et al., 1968, for species is therefore not usually fulfilled. The grouping of individual strains into the same kind or species is usually settled without benefit of the interfertility test. Moreover the mutability and the rapid growth of these organisms, combined with strong selection pressures from changes in the

environment, often leads to striking changes in appearance of a strain during its cultivation in the laboratory (Davis, et al., 1968).

Current dogma (Emmons, Binford and Utz, 1963; Conant, et al., 1954) contends that the genus Histoplasma contains no more than three species: H. capsulatum, H. duboisii, and H. farcinimosum. Some workers contend (Negroni, 1965) that H. duboisii should not be included in the same genus with H. capsulatum.

It is apparent from the results of experiments reported in this thesis that strain differences exist among the species of H. capsulatum. The first of the differences is morphological. The four strains pictured in the results (Figure 2) show the differences between strains on Sabouraud's dextrose agar with phosphate. Not pictured are the differences in morphology of the same strain grown on the five different media employed in this study. Careful observation of the same strain on different media made it apparent that significant variations occurred in macroscopic characteristics of the strain.

These characteristics might be related to the nitrogen source contained in the medium. The carbohydrate source was dextrose in each medium. The nitrogen source varied: 1) Sabouraud's dextrose agar with phosphate contained

peptone, 2) modified Sauton's contained asparagine, 3) synthetic 1 contained $(NH_4)_2SO_4$, 4) synthetic 2 contained asparagine and 5) synthetic 3 contained no nitrogen source. Sauton's and synthetic 2 contained the same nitrogen source, but macroscopically the colonies appeared different. Factors other than carbohydrate and nitrogen sources must play a role in the color and texture of the colony.

The mycelial phase of H. capsulatum grows well at 25° C on synthetic media having a single nitrogen source, although some strains demonstrate individual amino acid requirements (Rowley and Pine, 1955). Of the carbohydrate sources, dextrose gives optimal growth (Scheff, 1945). The growth on synthetic 1 was less in comparison to growth on Sabouraud's and Sauton's. Synthetic 3 supported growth which was scant, delicate and about the size of a dime. This latter medium contained no added nitrogen. The endogenous nitrogen present in the medium and the nitrogen present in the inoculum was apparently a limiting growth factor. Growth on synthetic medium 2 was similar in size to growth on Sabouraud's with phsophate; however, growth on synthetic 2 medium differed in color and texture.

In addition to the differences noted above, there are differences in the sporulation characteristics of each strain of H. capsulatum and growth on the various media.

Apparently there are certain limitations of interpretation with regard to the sporulation data due to the methods of study. It is probable that measurements made from colonies grown on media other than those studied might have given different results. It might also have been better to have emulsified the whole culture and then measured the numbers and sizes of the spores rather than selecting an arbitrary area as was done. It does not appear, however, that these criticisms would limit comparison between strains where all were treated in like manner.

The measurements of the spore sizes suggest that there are differences among media which induce differences in spore sizes. For example, macroconidia grown on Sabouraud's with phosphate were larger than the spores observed in the remaining media employed in the study. Mycelial phase cultures grown on Sauton's medium produced microconidia which were larger than those formed on the other media. The difference in size of the macroconidia is more striking than is the difference in microconidial sizes on the six media utilized in this study. The figures showing the sizes of microconidia and macroconidia of different strains of H. capsulatum yielded no overlapping between the two spore types. However, these figures represent only three of the six media studied, i.e., synthetic media 1, 2, and 3. These

media contained similar constituents except for the additional nitrogen source or lack of nitrogen. Apparently there was a size difference in macroconidia formed on synthetic 1 as compared to synthetic 3 media. Analysis of the remainder of the data yielded no additonal information.

Although sporulation of H. capsulatum is primarily a strain dependent phenomenon (Howell, 1939), some investigators have noted variation in degree of sporulation in supplemented media. For example, addition of phsophate ion to Sabouraud's medium (Artis and Baum, 1963) and variations of media containing different nitrogen or carbohydrate sources (Negroni, 1940) have been noted to enhance sporulation. The data that have been presented in this thesis suggest that there is a correlation between nutritional properties and sporulation ability.

The factor(s) or characteristic(s) of the culture media tested which contributed to the production of either macroconidia or microconidia were not examined. The enrichment of Sabouraud's medium with a source of phosphate such as KH_2PO_4 was reported by Artis and Baum (1963) as being useful in stimulating tuberculate spore production (macroconidia production). The results of their study demonstrated that tuberculate macroconidia are not in a resting or survival stage since depleted media and nutritionally poor media did

not stimulate spore production. Using calculations from the sporulation numbers, the Sabouraud's medium with additional phosphate ion supported relatively more macroconidia. Similar calculations suggested that the synthetic media 1 and 2, and modified Sauton's medium produced relatively more microconidia. Synthetic medium 3 supported formation of approximately equal numbers of both spore types.

Smith and Furcolow (1964) suggested that the addition of an infusion of starling manure to soil has the effect of producing more total particles and of producing a large number of microconidia as well as increasing the viability at least twice that observed in other media. Of particular interest was the finding that although large numbers of particles are produced on Sabouraud's medium, 84% of these particles are hyphal elements and not spores, but the overall viability was only 3%. It is clear that some essential element(s) in the extract of bird manure augments the sporulation potential of the organism in soil. However, the presence of large amounts of extraneous materials makes the use of this medium undesirable for immunological work with the derived mycelial phase.

Smith (1964) has reported that a medium composed of 0.6% yeast extract and 2% agar in distilled water stimulated rapid growth and sporulation of H. capsulatum similar to

that of starling manure extract medium. The rapid growth consisted of many viable microconidia and a low ratio of vegetative mycelium. These findings suggest that the spore-stimulating substances are nutritional in nature.

The results of sporulation experiments reported in this thesis support the premise that sporulation is related to the nutritional environment on which the H. capsulatum is grown. The measurement of the ability of each strain to produce spores was based on microscopic appearance of the mycelium and spores. The measurement was relative and rank statistics were used in assigning a number value to a set of defined terms. Using this method the sporulation number of each strain on each medium was obtained. This sporulation number gives no indication of the abundance of the spores but only the ability of each strain to produce spores. The sporulation number was used to indicate the ability of a strain to produce spores and the type of spore for preparation of vaccines. Sabouraud's medium with phosphate ion was chosen as the medium upon which the mycelial phase organisms were to be grown. This medium was chosen from among the media studied for the following reasons: 1) ease of preparation, 2) abundance of growth, and 3) ability to support spore production in abundant numbers. Harvest of the mycelial phase of H. capsulatum occurred after 10 weeks.

This period was chosen because maximum growth and numerous spores were obtained at this time.

Data from the experiments described in this thesis suggest that sporulation by H. capsulatum is dependent upon the strain of the organism and the medium on which it grows. Some strains will sporulate in such a manner that only well defined large macroconidia are produced (strain G10). Other strains, for example 3330 and 6651, formed large numbers of microconidia and few macroconidia. In many strains there is a marked size and morphological differentiation between microconidia and macroconidia. In a few strains the gradation of size of the spores progresses so smoothly from microconidia to macroconidia that no real distinction can be made between the two forms.

A fundamental problem in medical mycology is the question of variation in virulence between strains of H. capsulatum. Drouhet and Schwarz (1956) and Howell and Kipkie (1950) have reporte these differences in strains of H. capsulatum. In only one available report (Rowley and Huber, 1955) has it been observed that different strains of this organism were not significantly different with regard to mouse virulence. Using methods described in this thesis, significant differences in virulence were noted among the tested strains of H. capsulatum, i.e. an LD_{50} for

strain 3154 of 3.0×10^5, an LD_{50} for strain G17M of 1.1×10^6, and for strain 2813 of 3.0×10^8 (Table 13). It can be recalled that yeast phase organisms of each strain were injected intravenously. These differences in LD_{50} could be due to differences in the mouse virulence of the strains of organisms used but this difference might be unrelated to human disease. There was no morphological or physiological explanation apparent for the wide variance in mouse virulence observed with the different strains.

The 23 strains of H. capsulatum studied were isolated from human and animal sources, and from soils. The strains differed in mycelial phase morphology both in macroscopic colony appearance and in microscopic appearance. The yeast phase cells of the organisms observed were morphologically similar.

Results showed that the virulence of strain G17M did not change to any significant degree when maintained in the yeast phase on antibiotic containing blood agar for long periods of time at a temperature of 37° C. Similar results were demonstrated with strain 3154 but over a 1.5 year time period as opposed to 15 year period for G17M. Furthermore, other factors such as rapid passage on cultural medium, mouse passage, human passage, and conversion did not change the virulence of strain G17M.

The susceptibility of the mouse to infection by small doses of H. capsulatum, either spores or yeast phase, makes this host a significant one in which to study problems in immunity. Lethal infections within relatively short periods are produced only by parenteral inoculation of 10^5 or more yeast phase organisms depending on the virulence of the strain used. Since a mortality endpoint has certain advantages, challenge doses of strain 3154 were employed with doses as great in magnitude as 10^7 organisms administered by the intravenous route, that is, about 100 LD_{50} doses.

The strains of H. capsulatum utilized in immunity studies were chosen because of the following characteristics: strain G10 produced abundant macroconidia on Sabouraud's medium with phosphate. Strain G75 became pleomorphic after cultivation in the stock culture collection. Pleomorphism is defined in mycology as a degenerative change in a fungus that converts the colony into one that is completely void of characteristic spores required for identification (Ajello, et al., 1963). Pleomorphism is apparently irreversible. Strain G75 produced no spores, only mycelium. Strain 3330 exhibited good growth on Sabouraud's with phosphate ion, many macroconidia and few microconidia were formed. This same strain on synthetic medium 1 showed less growth than on Sabouraud's, but the number of microconidia formed was

higher. Because of the greater amount of growth on Sabouraud's with phosphate, this medium was selected to grow this strain of H. capsulatum. Strain 2645 of H. capsulatum was chosen because it produced an abundance of both types of spores. Strain G17M was chosen to represent the species in the yeast phase because of previous characterization of the organism at the University of Utah.

The intravenous route of challenge for infection of mice was chosen because of its superiority to other routes of infection for producing deaths in mice.

For our studies intracerebral injections have not been used because of the following problems: 1) a meningitis is produced which is rarely found in hosts naturally infected or in animals experimentally infected via other routes; 2) the volume of inoculum must necessarily be small; 3) the location of injections is not exact and deaths may be produced by focal brain lesions; 4) intracerebral injections are not made easily in most other species of laboratory animals.

The intraperitoneal route of immunization was used. Previous workers (Hill and Marcus, 1959) had found the intraperitoneal route as effective as intramuscular, subcutaneous or intravenous inoculations. The potency ratio for the comparison of normal groups to the other four groups

was found to be 2.4 with 95% confidence limits of 1.25 to 45.

In this thesis data were presented which permitted calculation of an estimate of the extent of resistance induced by immunization. This estimate is defined as the potency ratio, i.e., the ratio of the LD_{50} estimates for the control and to that of the immunized groups. The graphic method employed was modified by Litchfield and Wilcoxon (1949) from a method previously described (Miller and Tainter, 1944) for estimating lethal dose 50 and standard deviation. The procedure involved fitting dose-response lines by eye and calculation of LD_{50} and its error. By means of graphs and nomograms potency ratios and their errors were calculated. Challenge of the accuracy of the method by Finney (1952) was effectively answered by Litchfield and Wilcoxon (1953).

Since the method of analysis was based on the assumption of a dose-response relationship, the adaptation to data presented here was valid only if there existed a direct relationship between challenge dose of organisms and mortality. Although the dose-response characteristic following organism challenge was clearly evident for normal mice, this parameter was less apparent for the immunized animals. The data have indicated that a challenge dose-mortality response relationship did exist with immunized as well as normal mice: therefore the application of the method of analysis was

justified. However, the shallow slopes of dose-response lines lead to a wide range in the confidence limits.

Intraperitoneal vaccination of the mice with cell free extract of strain G10, a strain whose sporulation number indicated macroconidia production, and intravenous challenge (administered ten days postvaccination) gave the following potency ratios with the vaccination amounts in milligrams of dry weight: 3 mg, 31; 1 mg, 24; 0.4 mg, 18.5; and 0.1 mg, 5.0. Analysis of the potency ratios for this strain depended on the finding that the LD_{50} values of the saline controls were 4.2×10^5 and 7.0×10^5. There was a definite dose-response relationship between the potency ratios. Careful observation of the mortality ratios comparing saline controls and various immunized groups yielded other dose-response relationships. Finally, analysis of results of culture of organs from survivors showed a dose-response dependence. From this information one could deduce that as the dose of the vaccine is increased the mortality ratio, per cent deaths, culture results decrease and the LD_{50} values increase, i.e., it required greater numbers of organisms as a challenge dose to achieve the same effect. For example at a challenge dose of 1×10^6 the per cent deaths in the saline control group was 38% and it was also 38% for the group immunized with 0.1 mg dry weight cell

free extract. For the group immunized with 0.4 mg of the cell free extract, the deaths reached 11%. Looking at a challenge dose of 1×10^7, the per cent deaths in the saline control group was 100%, 20% for 0.8 mg, 45% for 0.4 mg and 60% for 0.1 mg. The per cent culture positive data yielded similar results as follows: 60% for 0.8 mg, 90% for 0.4 mg and 100% for 0.1 mg.

In animals vaccinated with 7.3×10^4 whole macroconidia of strain G10 the potency ratio was 3.4. This ratio was smaller than any of the values obtained with the cell free extract vaccine. The comparison can be made only with the understanding that the dry weight of the whole macroconidia was unknown.

Similar dose-response relationships were observed in the vaccination with strain G75. The potency ratios that could be calculated were for 2 mg a value of 20; for 0.4 mg, 4.7 and for 0.1 mg a value of 10. The dose-response relationship was unapparent in this case since the 0.1 mg ratio was higher than the 0.4 mg.

Strain 3330 and strain 2645 of H. capsulatum yielded similar dose-response relationships with the potency ratios increasing as the dry weight of the vaccine increased.

The cell free extract of yeast phase cells of strain G17M yielded potency ratios of 23 for 0.4 mg and 11.5 for

0.1 mg. Whole formalin-killed yeast cells used as a vaccine gave potency ratios of 7.2 for 10^6 cells and 11.4 for 5×10^7 cells. Hill and Marcus (1959) using a vaccine prepared from 4×10^7 cells prepared from strain G17M or 6651, obtained a potency ratio of 5.3 (1.21-23) when the mice were challenged with G17M. These two results do not necessarily conflict since the immunization procedures used differ and also the strain of H. capsulatum used to challenge the immunized mice. Knight and Marcus (1958), in animals vaccinated with approximately 10^7 whole killed organisms, found a potency ratio of 7.6. The difference in the data reported in this thesis, between 1×10^6 cells and 5×10^7 cells, i.e. approximately a 50 fold increase in the number of cells used to immunize, deserves further study.

Two of the strains of H. capsulatum studied, G10 and G17M, had similar potency ratios with regard to vaccine prepared from cell free extract. Strain G10 had a potency value of 18.5 (6-57) for 0.4 mg, whereas strain G17M had a potency ratio of 23 (7.8-69) for 0.4 mg. Values were similar for 0.1 mg dry weight of vaccine: strain G10, 5.0 (1.6-15) and strain G17M, 11.5 (3.6-38). The vaccine prepared from strain G10 was from the mycelial phase and according to the sporulation number this strain produced mainly macroconidia. The vaccine from strain G17M was

prepared from the yeast phase of the organism. Using the carbohydrate to protein ratio, strain G10 and strain G17M had similar chemical composition. Strain G10 had a ratio of 57 and strain G17M a ratio of 56. There was a suggestion of a relationship between chemical composition and immunizing capacity.

Knight, Hill and Marcus (1959) studied the immunogenicity of a vaccine prepared from a polysaccharide moiety of the yeast phase growth of H. capsulatum. The polysaccharide was capable of stimulating active resistance against intravenously induced histoplasmosis in mice. Following intraperitoneal vaccination of mice with graded doses or a single dose, both of which totaled 1 mg of polysaccharide, the potency ratio for both groups was approximately 4.3.

The remaining mycelial phase vaccines prepared from strains G75, 2645, and 3330 had low carbohydrate to protein ratio, i.e., the percentage of protein found in these vaccines was higher. Strain G75 had both the highest carbohydrate and protein percentage.

The relationship between dry weight, protein, and polysaccharide content of the mycelial phase cell walls of H. capsulatum has been previously reported by McNall (1962). The protein content was 11.2% of the dry weight of isolated cell walls. The polysaccharide was reported as 30.0%.

Studies on six strains of yeast phase H. capsulatum by Pine, Boone and McLaughlin (1966) showed protein content ranging from 7-38% and carbohydrate content ranging from 7-31%.

The values for cell free extracts of mycelial phase of the fungus reported in this thesis ranged from 33-47% carbohydrate for four strains. The protein content ranged from 0.6% to 7.1% based on dry weight of the mycelial phase. The yeast phase cell free extract of G17M contained 39% carbohydrate and 0.7% protein whereas the whole cells from the same strain were composed of 39% carbohydrate and 3.2% protein.

SUMMARY

Twenty-three strains of H. capsulatum were characterized as to: 1) macroscopic appearance of the fungus on various media; 2) differential spore production on various media; 3) intravenous LD_{50} of the yeast phase of certain strains of the organism; and 4) immunogenic capacity of vaccines prepared from various elements of the selected strains of the fungus.

Results of experiments reflected apparent strain differences which exist among the species H. capsulatum. The first difference was morphological and might be related to the nitrogen source contained in the media. Sabouraud's medium with phosphate supported the best growth. The remainder of the media supported mycelial growth in the following comparative order from good to scant: modified Sauton's, synthetic 2, synthetic 1, and synthetic 3. Synthetic 3 supported growth which was scant and delicate.

Variations were also noted in the sporulation characteristics of each strain grown on the various media. The measurements of spore sizes were consistant with values reported by other workers. Sabouraud's medium with phosphate produced larger macroconidia than the other media studied. Larger microconidia were produced on Sauton's medium than spores formed on the remaining media. The macroconidial

and microconidial spores ranged in size from 6.5 to 14.3 u and 2.3 to 5.8 u respectively. The relative measurement of numbers of spores produced by each strain were based on a rank system. Strain G10 and 2870 produced a predominance of macroconidia as indicated by the sporulation number. Two of the 23 strains (G75 and 2586) produced only microconidia. Two strains, G17M and 2888, produced equal numbers of both spore types. The remaining strains yielded sporulation numbers that varied about those of the above described strains. The medium which produced the macroconidia best was Sabouraud's with phosphate. Synthetic 1 and 2, and modified Sauton's produced mainly microconidia. The remaining medium, synthetic medium 3, produced sparse but equal numbers of both spore types.

The intravenous LD_{50} of selected yeast phase strains of H. capsulatum were studied. Significant differences in virulence were noted. Strain 3154 had an LD_{50} value of 3.0×10^5. At the other extreme, the LD_{50} for strain 2813 was 3.0×10^8. The values of the remaining strains fell between these two LD_{50} values. The yeast phase of strain 3154 was used as the challenge organism in the immunization experiments.

Vaccines prepared from G10, G17M, 2645, and 3330 were used to imunize mice. After challenge with the yeast phase of 3154, the potency ratios were determined. The vaccine

prepared from the mycelial phase of G10 cell free extract and the cell free extract prepared from the yeast phase of G17M had similar potency ratios. These two vaccines, G10 and G17M had similar chemical composition also, with high carbohydrate and low protein content. The remaining vaccines, prepared either from cell free extracts or whole cells, yielded potency ratios that varied from 1.5 to 20.0, with no correlation between chemical composition and potency ratio.

The 23 strains of H. capsulatum studied were isolated from human and animal sources, and from soils. The strains differed in mycelial phase morphology, mouse virulence, and immunogenic capacity as determined by the results of these experiments.

BIBLIOGRAPHY

Ajello, L., and S. Cheng. 1967. Sexual reproduction in Histoplasma capsulatum. Mycologia 59:689-697.

Ajello, L., L. K. Georg, W. Kaplan, and L. Kaufman. 1963. Laboratory Manual for Medical Mycology. U. S. Government Printing Office, Washington, D. C.

Artis, D., and G. L. Baum. 1963. Tuberculate spore formation by thirty-two strains of Histoplasma capsulatum. Mycopathologia et Mycologia Applicata 21:29-35.

Blank, F. 1954. On the cell walls of dimorphic fungi causing systemic infections. Canadian Journal of Microbiology 1:1-5.

Box, E. D., and N. T. Briggs. 1961. Endotoxin susceptibility and delayed hypersensitivity in experimental histoplasmosis. Journal of Immunology 87:485-491.

Brown, J. H., K. M. Cook, F. G. Ney, and T. Hatch. 1950. Influence of particle size upon the retention of particle matter in the human lung. American Journal of Public Health 40:450-458.

Christie, A., and T. C. Peterson. 1945. Pulmonary calcifications in negative reactors to tuberculin. American Journal of Public Health 35:1131-1147.

Ciferri, R., and P. Redaelli. 1934. Histoplasma capsulatum Darling, the agent of histoplasmosis. Systematic position and characteristics. Journal of Tropical Medicine and Hygiene 37:278-280.

Conant, N. F. 1941. A cultural study of the life cycle of Histoplasma capsulatum Darling (1906). Journal of Bacteriology 41:563-579.

Conant, N. F., D. T. Smith, R. D. Baker, J. L. Callaway, and D. S. Martin. 1954. Manual of Clinical Mycology. W. B. Saunders Co., Philadelphia, Pa.

Converse, J. L., M. W. Castleberry, A. R. Besemer, and E. M. Snyder. 1962. Immunization of mice against coccidioidomycosis. Journal of Bacteriology 84:46-52.

Cooke, W. B., and P. W. Kabler. 1953. The survival of Histoplasma capsulatum in water. Lloydia 16:252-256.

Cozad, G. C., and M. L. Furcolow. 1953. Laboratory studies of Histoplasma capsulatum. II. Size of the spores. Journal of Infectious Disease 92:77-84.

Cross, F. W. 1948. The effect of hydrogen ion concentration of the yeast-like phase of Histoplasma capsulatum (Darling). Public Health Reports (U.S.) 63:739-746.

Darling, S. T. 1906. A protozoan general infection producing pseudotubercles in the lungs and focal necrosis in the liver, spleen, and lymph nodes. Journal of the American Medical Association 46:1283-1285.

Darling, S. T. 1908. Histoplasmosis: a fatal infectious disease resembling kala-azar found among natives of tropical America. Archives of Internal Medicine 2:107-123.

Darling, S. T. 1909. The morphology of the parasite (Histoplasma capsulatum) and lesions of histoplasmosis, a fatal disease of tropical America. Journal of Experimental Medicine 11:515-531.

Da Rocha-Lima, H. 1912. Histoplasmosis und epizootische lymphangitis. Archiv fur schiffs - und Tropenhygiene 16:79-85.

Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, Jr. 1968. Microbiology. Harper and Row Incorp., New York.

De Monbreun, W. A. 1934. Cultivation and cultural characteristics of Darling's Histoplasma capsulatum. American Journal of Tropical Medicine 14:93-125.

Dodd, K., and E. H. Tompkins. 1934. A case of histoplasmosis of Darling in an infant. American Journal of Tropical Medicine 14:127-137.

Drouchet, E., and J. Schwarz. 1956. Comparative studies with 18 strains of Histoplasma. Morphology in tissues and virulence of African and American strains. Journal of Laboratory and Clinical Medicine 47:128-139.

Emmons, C. W., C. H. Binford, and J. P. Utz. 1963. Medical Mycology. Lea and Febiger, Philadelphia, Pa.

Emmons, C. W., H. B. Morlan, and E. L. Hill. 1949. Isolation of Histoplasma capsulatum from soil. Public Health Reports (U.S.) 64:1423-1430.

Farrell, R. L., C. R. Cole, J. A. Prior, and S. Saslaw. 1953. Experimental histoplasmosis. I. Methods for production of histoplasmosis in dogs. Proceedings of the Society for Experimental Biology and Medicine 84:51-54.

Finney, D. J. 1952. Graphic estimation of relative potency from quantal responses. Journal of Pharmacology and Experimental Therapy 104:440-444.

Friedman, L., and C. E. Smith. 1956. Vaccination of mice against Coccidioides immitis. American Review of Tuberculosis and Pulmonary Diseases 74:245-248.

Friedman, L., C. E. Smith, and L. E. Gordon. 1955. The assay of virulence of Coccidioides in white mice. Journal of Infectious Disease 97:311-316.

Furcolow, M. L. 1960. Epidemiology of histoplasmosis, p. 113-148. In H. C. Sweany (ed.), Histoplasmosis, C. C. Thomas, Springfield, Ill.

Furcolow, M. L. 1965. Environmental aspects of histoplasmosis. Archives of Environmental Health 10:4-10.

Grayston, J. T., and P. Altman. 1954. The pathogenesis and pathology of experimental histoplasmosis infections in mice. Journal of Laboratory and Clinical Medicine 44:804-805.

Grayston, J. T., C. G. Loosli, and E. R. Alexander. 1951. The isolation of Histoplasma capsulatum from soil in an unused silo. Science 114:323-324.

Grayston, J. T., and S. B. Salvin. 1956. Experimental histoplasmosis in immunized and nonimmunized mice. Comparative pathology. Archives of Pathology 61:422-433.

Hansmann, G. H., and J. R. Schenken. 1934. Unique infection in man caused by new yeast-like organism, pathogenic member of genus Sepedonium. American Journal of Pathology 10:731-738.

Hasenclever, H. F., and W. O. Mitchell. 1963. Acquired immunity to candidiasis in mice. Journal of Bacteriology 86:401-406.

Hedgecock, L. W. 1961. Effect of vaccines prepared from Histoplasma capsulatum and other yeasts on experimental tuberculosis. Journal of Bacteriology 82:115-123.

Hill, G. A. 1958. Host resistance in systemic mycotic infections. Ph.D. thesis. University of Utah.

Hill, G. A., and S. Marcus. 1959. Resistance induced against Histoplasma capsulatum, quantitative aspects. Journal of Infectious Disease 105:26-30.

Hill, G. A., and S. Marcus. 1960. Study of cellular mechanisms in resistance to systemic Histoplasma capsulatum infection. Journal of Immunology 85:6-13.

Howard, D. N. 1964. Intracellular behavior of Histoplasma capsulatum. Journal of Bacteriology 87:33-38.

Howard, D. N. 1965. Intracellular growth of Histoplasma capsulatum. Journal of Bacteriology 89:518-523.

Howell, A., Jr. 1939. Studies on Histoplasma capsulatum and similar form species. I. Morphology and development. Mycologia 31:191-216.

Howell, A., Jr. 1940. Studies on Histoplasma capsulatum and similar form species. II. Effect of temperature. Mycologia 32:671-680.

Howell, A., Jr. 1941. Studies on Histoplasma capsulatum and similar form species. III. Effect of hydrogen in concentration. Mycologia 33:103-117.

Howell, A., Jr. 1948. The efficiency of methods for the isolation of Histoplasma capsulatum. Public Health Reports (U.S.) 63:173-178.

Howell, A., Jr., and G. F. Kipkie. 1950. Studies on experimental histoplasmosis. IV. A comparison of the virulence of five strains of Histoplasma capsulatum by intracerebral inoculation of male DBA line 1 mice. Journal of Laboratory and Clinical Medicine 36:547-554.

Johnson, R. W., and M. Scherago. 1960. The effect of histoplasmin in vitro on the migration of leucocytes from guinea pigs experimentally infected with Histoplasma capsulatum. American Review of Respiratory Disease 81:96-99.

Kabat, E. A., and M. Mayer. 1962. Experimental Immunochemistry, 2nd ed. C. C. Thomas, Springfield, Ill.

Kaufman, L., and S. Blumer. 1966. Occurrence of Serotypes among Histoplasma capsulatum strains. Journal of Bacteriology 91:1434-1439.

Knight, R. A., G. Hill, and S. Marcus. 1959. Immunization of mice with polysaccharides of Histoplasma capsulatum. Proceedings of the Society of Experimental Biology and Medicine 100:356-358.

Knight, R. A., and S. Marcus. 1958. Polysaccharide skin test antigens derived from Histoplasma capsulatum and Blastomyces dermatitidis. American Review of Tuberculosis and Pulmonary Disease 77:983-989.

Kong, Y. M., and H. B. Levine. 1967. Experimentally induced immunity in the mycoses. Bacteriology Reviews 31:35-53.

Kong, Y. M., H. B. Levine, and C. E. Smith. 1963. Immunogenic properties of nondisrupted and disrupted spherules of Coccidioides immitis in mice. Sabouraudia 2:131-142.

Kwon-Chung, K. J. 1968. Gymnoascus demonbreunnii Ajello and Cheng: Evidence that it is not the perfect state of Histoplasma capsulatum Darling. Sabouraudia 6: 168-175.

Langeron, M., and R. Vanbreuseghem. 1965. Translated by J. Wilkinson. Outline of Mycology. Pitman and Sons, Ltd., London.

Larsh, H. W. 1960. Natural and experimental epidemiology of histoplasmosis. Annals of the New York Academy of Sciences 89:78-90.

Larsh, H. W., and G. C. Cozad. Respiratory infection of mice with Histoplasma capsulatum. Mycopathologia et Mycologia Applicata 27:305-310.

Levine, H. B., J. M. Cobb, and C. E. Smith. 1960. Immunity to coccidioidomycosis induced in mice by purified spherule, arthrospore, and mycelial vaccines. Transactions of the New York Academy of Sciences 22:436-449.

Levine, H. B., J. M. Cobb, and C. E. Smith. 1961. Immunogenicity of spherule-endospore vaccines of Coccidioides immitis for mice. Journal of Immunology 87:218-227.

Levine, H. B., Y. M. Kong, and C. E. Smith. 1965. Immunization of mice to Coccidioides immitis: dose, regimen, and spherulation stage of killed spherule vaccines. Journal of Immunology 94:132-14.

Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluation of dose-effect experiments. Journal of Pharmacology and Experimental Therapeutics 96:99-113.

Litchfield, J. T., and F. Wilcoxon. 1953. The reliability of graphic estimates of relative potency from dose-per cent effect curves. Journal of Pharmacology and Experimental Therapeutics 108:18-25.

Marcus, S., Y. Aoki, and G. A. Hill. 1965. Delayed skin test reactions induced by polysaccharide from coccidioidin. Federation Proceedings 24:182.

Marcus, S., and F. R. Rambo. 1952. Reproducible systemic mycotic infection in mice. Bacteriology Proceedings, p. 88.

Markowitz, H. 1964. Polysaccharide antigens from Histoplasma capsulatum. Proceedings of the Society of Experimental Biology and Medicine 115:697-700.

McNall, E. G. 1962. Cell wall constituents of pathogenic fungi. P. 139-147. In G. Dalldorf (ed.) Fungi and Fungus Diseases. C. C. Thomas, Springfield, Ill.

McVeigh, I., and K. Morton. 1965. Nutritional studies of Histoplasma capsulatum. Mycopathologia et Mycologia Applicata 25:294-308.

Menges, R. W., M. L. Furcolow, H. W. Larsh, and A. Hinton. 1952. Laboratory studies on histoplasmosis. I. The effect of humidity and temperature on the growth of Histoplasma capsulatum. Journal of Infectious Disease 90:67-70.

Miller, L. C., and M. L. Tainter. 1944. Estimation of the LD_{50} and error by means of logarithmic probit graph paper. Proceedings of the Society of Experimental Biology and Medicine 57:261-264.

Miya, F., and S. Marcus. 1961. Effect of humoral factors on in vitro phagocytic and cytopeptic activities of normal and "immune" phagocytes. Journal of Immunology 86:652-668.

Negroni, P. 1940. Estudo micrologico del primer caso Sud-Americans de histoplasmosis. Revista Instituto Bacteriologico 9:239-294.

Negroni, P. 1965. Histoplasmosis. C. C. Thomas, Springfield, Ill.

Nielsen, G. E., and R. E. Evans. 1954. A study of the sporulation of Histoplasma capsulatum. Journal of Bacteriology 68:261-264.

Okudaira, M., and J. Schwarz. 1962. Experimental ocular histoplasmosis in rats. A histopathologic study of immunogenic and hypersensitive ophthalmitis produced in rat by Histoplasma capsulatum. American Journal of Ophthalmology 54:427-444.

Palmer, C. E. 1945. Nontuberculous pulmonary calcifications and sensitivity to histoplasmin. Public Health Reports (U.S.) 60:513-520.

Pappagianis, D., H. B. Levine, C. E. Smith, R. J. Berman, and G. S. Kobayashi. 1961. Immunization of mice with viable Coccidioides immitis. Journal of Immunology 86:28-34.

Pappagianis, D., R. L. Miller, C. E. Smith, and G. S. Kobayashi. 1960. Response of monkeys to respiratory challenge following subcutaneous inoculation with Coccidioides immitis. American Review of Respiratory Diseases 82:244-250.

Pappagianis, D., C. E. Smith, and G. S. Kobayashi. 1956. Relationship of the in vivo form of Coccidioides immitis to virulence. Journal of Infectious Disease 98:312-319.

Pine, L. 1954. Studies on the growth of Histoplasma capsulatum. I. Growth of the yeast in liquid media. Journal of Bacteriology 68:671-679.

Pine, L. 1955. Studies on the growth of Histoplasma capsulatum. II. Growth of the yeast phase on agar media. Journal of Bacteriology 70:375-381.

Pine, L. 1957. Studies on the growth of Histoplasma capsulatum. III. Effect of thiamin and other vitamins on the growth of the yeast and mycelial phases of Histoplasma capsulatum. Journal of Bacteriology 74:239-245.

Pine, L. 1960. Morphological and physiological characters of Histoplasma capsulatum, p. 40-67. In H. C. Sweany (ed.) Histoplasmosis. C. C. Thomas, Springfield, Ill.

Pine, L., C. J. Boone, and D. McLaughlin. 1966. Antigenic properties of the cell wall and other fractions of the yeast form of Histoplasma capsulatum. Journal of Bacteriology 91:2158-2168.

Pine, L., and C. L. Peacock. 1958. Studies on the growth of Histoplasma capsulatum. IV. Factors influencing the conversion of the mycelial phase to the yeast phase. Journal of Bacteriology 75:167-174.

Procknow, J. J., M. I. Page, and C. G. Loosli. 1960. Early pathogenesis of experimental histoplasmosis. Archives of Pathology 69:413-426.

Rambo, F. R., S. Marcus, and F. D. Gunn. 1955. Variations in virulence for mice of systemic mycotic organisms in relation to reinfection effects. Federation Proceedings 14:475.

Ribi, E., and S. B. Salvin. 1956. Antigens from the yeast phase of Histoplasma capsulatum. I. Morphology of the cell as revealed by the electron microscope. Experimental Cell Research 10:394-404.

Ritter, R. C. 1954. Studies on the viability of Histoplasma capsulatum in tap water. American Journal of Public Health 44:1299-1304.

Rowley, D. A., and M. Huber. 1955. Pathogenesis of experimental histoplasmosis in mice. II. Comparison of the intravenous and intraperitoneal routes of infection: comparison of the pathogenicity of four strains of Histoplasma capsulatum. Journal of Infectious Disease 97:27-34.

Rowley, D. A., and M. Huber. 1956. Growth of Histoplasma capsulatum in normal, superinfected, and immunized mice. Journal of Immunology 77:15-23.

Rowley, D. A., and L. Pine. 1955. Some nutritional factors influencing growth of yeast cells of Histoplasma capsulatum to mycelial colonies. Journal of Bacteriology 69:695-700.

Salfelder, K., and F. Schwartz. 1964. Cross reaction to Histoplasma capsulatum in mice. Sabouraudia 3:164-166.

Salvin, S. B. 1947. Cultural studies on the yeast-like phase of Histoplasma capsulatum Darling. Journal of Bacteriology 54:655-660.

Salvin, S. B. 1949. Cysteine and related compounds in the growth of the yeast-like phase of Histoplasma capsulatum. Journal of Infectious Disease 84:275-283.

Salvin, S. B. 1953. Immunization of mice against Histoplasma capsulatum. Journal of Immunology 70:267-270.

Salvin, S. B. 1955a. Further studies on immunization of mice against Histoplasma capsulatum. Americal Journal of Hygiene 61:72-81.

Salvin, S. B. 1955b. Resistance to reinfection in experimental histoplasmosis. Journal of Immunology 74:214-221.

Salvin, S. B. 1958. The influence of leucocytes from sensitized mice on resistance to Histoplasma capsulatum. American Journal of Hygiene 68:233-241.

Salvin, S. B. 1960. Resistance of animals and man to histoplasmosis, p. 99-112. In H. C. Sweany (ed.) Histoplasmosis. C. C. Thomas, Springfield, Ill.

Salvin, S. B. 1963. Immunologic aspects of the mycoses. Progress in Allergy 7:213-331.

Salvin, S. B. 1965. Constituents of the cell wall of the yeast phase of Histoplasma capsulatum. American Review of Respiratory Diseases 92:119-125.

Salvin, S. B., and E. Ribi. 1955. Antigens from yeast phase of Histoplasma capsulatum. II. Immunologic properties of protoplasm vs cell walls. Proceedings of the Society for Experimental Biology and Medicine 90:287-294.

Salvin, S. B., and R. F. Smith. 1959. Antigens from the yeast phase of Histoplasma capsulatum. III. Isolation, properties, and activity of a protein-carbohydrate complex. Journal of Infectious Disease 105:45-53.

Saslaw, S., H. N. Carlisle, and J. Sparks. 1960. Experimental histoplasmosis in monkeys. Proceedings of the Society for Experimental Biology and Medicine 103:342-344.

Saslaw, S., and J. Schaefer. 1956. Survival of Histoplasma capsulatum in experimental histoplasmosis in mice. Proceedings of the Society for Experimental Biology and Medicine 91:412-414.

Schaefer, J., and S. Saslaw. 1954. Some factors affecting resistance of mice to experimental histoplasmosis. Proceedings of the Society for Experimental Biology and Medicine 85:223-225.

Scheff, G. J. 1945. Biochemical and immunological properties of Histoplasma capsulatum. Yale Journal of Biological Medicine 18:41-54.

Scherr, G. H. 1956. The influence of temperature and -SH groups on the growth of dimorphic fungi. (abstract) Bacteriological Proceedings p. 85.

Scherr, G. H. 1957. Studies on the dimorphism of Histoplasma capsulatum. I. The roles of -SH groups and incubation temperature. Experimental Cell Research 12:92-102.

Smith, C. D. 1964. II. Evidence of the presence in yeast extract of substances which stimulate the growth of Histoplasma capsulatum and Blastomyces dermatitidis similarly to that found in starling manure extract. Mycopathologia et Mycologia Applicata 22:99-105.

Smith, C. D., and M. B. Furcolow. 1964. The demonstration of growth stimulating substances for Histoplasma capsulatum and Blastomyces dermatitidis in infusions of starling (Sturnis vulgaris) manure. Mycopathologia et Mycologia Applicata 22:73-80.

Stewart, R. A., and K. F. Meyer. 1932. Isolation of Coccidioides immitis (Stiles) from the soil. Proceedings of the Society for Experimental Biology and Medicine 29:937-938.

Willis, H. S., and M. M. Cummings. 1952. Diagnostic and Experimental Methods in Tuberculosis. C. C. Thomas, Springfield, Ill.

Wu, W. G., and S. Marcus. 1963. Humoral factors in cellular resistance, I. The effects of heated and unheated homologous and heterologous sera on phagocytosis and cytopepsis by normal and "immune" macrophages. Journal of Immunology 91:313-322.

VITA

Name	Kenneth L. Anderson
Birthplace	Jefferson, Iowa
Birthdate	April 30, 1940
Elementary and Secondary education	Jefferson, Iowa
Universities	Iowa State University Ames, Iowa 1958-1963 B.S. Botany; minors Zoology, Chemistry University of Iowa Iowa City, Iowa 1963-1965 M.S. Microbiology University of Utah Salt Lake City, Utah 1965-1968
Professional organizations	American Society for Microbiology Sigma Xi American Association for the Advancement of Science American Institute for Biological Sciences
Thesis title	M.S. University of Iowa "Immunologically Active Substances Produced *in vivo* by *Histoplasma Capsulatum*"

RESEARCH PROPOSALS

submitted

by

Kenneth Leroy Anderson

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Department of Microbiology

University of Utah

August, 1968

RESEARCH PROPOSALS

1. Investigation in depth should be undertaken of the chemical composition and morphologic and antigenic structure of the soluble, extracellular growth products, cell walls, membrane and cytoplasmic fractions of *Histoplasma capsulatum*.
2. The relationship of diagnostic and protective antigens with cell composition and structure of *H. capsulatum* remains unknown and requires study.
3. Further studies on the critical separation of the spores and mycelium of *H. capsulatum* would yield specific information with regard to the chemical composition of these elements of *H. capsulatum*.
4. The use of aerosol challenge, using purified spore and mycelial elements, might yield more specific information concerning the mechanism of infection in experimental pulmonary histoplasmosis.
5. Immunity studies utilizing vaccines produced from various elements of *H. capsulatum* and subsequent challenge of immunized animals with either purified viable microconidia or macroconidia would yield significant basic immuno-mycologic information.

6. Epidemiological studies on a significant sample of Utah residents to determine the incidence of skin test sensitivity to H. capsulatum and Blastomyces dermatitidis should be accomplished correlating skin test data with attempts to isolate these fungi.
7. Relatively few pertinent data are available to indicate the potential clinical usefulness of antifungal vaccination. Possibly such information could be obtained by immunity trials in a variety of hosts challenged under conditions that simulate or approximate natural exposure.
8. The significance of delayed hypersensitivity in the chronic fungal diseases in unknown and merits further study.
9. Studies must be pursued involving the relationship of the use of immunosuppressive drugs and opportunistic mycotic pathogens in patients that have received or are about to receive transplanted organs.
10. Further studies are indicated of the relationship of the various strains of H. capsulatum and the morphology, virulence, and spore production of these strains.